SIGILLVM · VNIVERSITATIS · CALIFORNIENSIS
FIAT LVX
MDCCCLXVIII
EX LIBRIS
Reese

UNIV. OF CALIFORNIA

THE

MICROTOMIST'S VADE-MECUM

A HANDBOOK OF THE METHODS OF MICROSCOPIC ANATOMY

REESE LIBRARY
OF THE
UNIVERSITY
OF CALIFORNIA

BY

ARTHUR BOLLES LEE

FOURTH EDITION

LONDON
J. & A. CHURCHILL
7, GREAT MARLBOROUGH STREET
1896

QH 231
L4
1896

71213

REESE LIBRARY
OF THE
UNIVERSITY
OF CALIFORNIA

PREFACE

The short period of three years that has elapsed since the publication of the last edition of this work has not brought with it any radical change in the methods of histological research. Such progress as has been realised has consisted rather in improvements in the detail of already-established methods than in the introduction of new methods or new reagents. Nevertheless, the present edition has undergone a most thorough revision—a revision indeed so thorough as to amount to extensive re-writing in many parts.

It has seemed to me advisable in the interest of the beginner, and indeed in the interest of readers who are not beginners at all, to enter more fully than was hitherto done into the detail of the more important processes, to explain more fully the principles on which they are founded, and to add in many cases a critical estimate of their rationality and practical value. In consequence of this re-writing, and in spite of strenuous efforts to keep down the bulk of the work, it has turned out to be considerably increased. I regret, however, this increase the less, in so far as it is due rather to the ampler treatment that has been accorded to the more valuable methods than to increase in the number of processes described. The number of new processes described is in fact a smaller one than I have had to deal with in the preparation of any edition since the first.

The classification of the various methods has received most careful attention, and has been in many cases greatly simplified, whilst at the same time a large number of superfluous processes have been rejected. Advice to the beginner concerning the choice of methods has been given wherever practicable, and I think that notwithstanding the abundance and complexity of the matters treated of, there can hardly be any risk that the student may be unable to see the wood for the trees.

The chapters treating of Staining and of the Carmine and Hæmateïn stains have had the great advantage of revision by Dr. Paul Mayer, who, it is superfluous to remind the reader, has made a speciality of this subject, with results brilliant alike in theory and in practice. Not indeed that the present English text has been directly revised by him, but that it has been prepared from a recent text so revised. Dr. Mayer was good enough to revise most carefully the three corresponding chapters prepared by me for the recent new edition of the *Traité des Méthodes Techniques de l'Anatomie Microscopique* (LEE et HENNEGUY), and in the preparation of the present English text I have closely followed the chapters so revised.

No less obligation have I to express to Professor van Gehuchten, who with great kindness has thoroughly revised for me the three chapters entitled Neurological Methods. It occurred to me that my treatment of this complicated subject could not but gain greatly by the advice of an observer who is not only one of the foremost of the new school of neurologists but at the same time an instructed and capable cytologist, and therefore likely to sympathise with my feeling that it would be much to be deplored that the study of nervous anatomy should degenerate into a mere study of topographical relations, to the neglect of the inner mechanism of nervous elements. By Professor van Gehuchten's advice I have entirely re-arranged the contents of these three chapters according to a scheme worked out by him, thereby effecting a great gain in clearness of exposition. I cannot but acknowledge that the arrangement adopted in previous editions resulted in something like a chaos; whilst the new arrangement may, I think, fairly claim to be natural, logical, and easily comprehensible. By his advice too I have entirely re-written the account of the bichromate of silver impregnations of Golgi; the account as it now stands is, I believe, the only complete one that has appeared in the English language.

I am under the greatest obligation to Professor van Gehuchten, as well as to Dr. Paul Mayer, for the generous assistance which enables me to affirm that the important subjects in question have been treated with all the requisite accuracy and thoroughness.

The essential feature of the first edition was that it was an altogether exhaustive collection of all the methods of preparation that had up to that time been recommended as useful for the purposes of microscopic anatomy, and its primary intention that of being a work of reference for the instructed anatomist. Its character of a guide to the beginner was secondary only. It contained indeed a general introduction and much explanatory matter in the different chapters, but, on the whole, the didactic matter bore but an insufficient proportion to the historical matter. This has now been rectified. It has come to pass that during the repeated operations of revision to which the book has been subjected, the explanatory and didactic element has been continually increasing, whilst at the same time the historical element has been continually diminishing—diminishing, that is, in all parts of the book relatively to the former element, and in some parts absolutely (as may be seen, for instance, by comparing the number of formulæ given in the chapters on carmine and hæmatoxylin with the number given in former editions). On the one hand the book has been lightened by the jettison of much useless matter, and on the other hand there has been accorded to the matter that has been retained a far ampler share than before of explanation and detail. To such an extent, indeed, have the instructions to students and other explanatory matter been amplified that I am not acquainted with any modern work on the subject that contains anything like so complete an account of the various fundamental operations of histological technique—fixing, imbedding, staining, and the like. I only felt justified in claiming for the first edition that it "went far to make up a formal treatise on the art." Through the changes abovementioned the book has come to assume altogether the character of a formal treatise, and now contains in due proportions both the grammar and the dictionary of the art.

The rejection of superfluous matter above referred to relates chiefly to old methods that have been before the public for so long a time that there can be no doubt that they have no good claim to further survival, whilst recent methods, which may be considered to be still on their probation, have been treated with the accustomed fulness.

NYON, SWITZERLAND;
September, 1896.

CONTENTS

PART I

CHAPTER I.

PAGE
INTRODUCTORY.—The General Method 1

CHAPTER II.

KILLING 11

CHAPTER III.

FIXING AND HARDENING 18

CHAPTER IV.

FIXING AGENTS: MINERAL ACIDS AND THEIR SALTS . . . 24

CHAPTER V.

FIXING AGENTS: CHLORIDES, ORGANIC ACIDS, AND OTHERS . . 38

CHAPTER VI.

HARDENING AGENTS 56

CHAPTER VII.

CLEARING AGENTS 64

CHAPTER VIII.

IMBEDDING METHODS.—INTRODUCTION 71

CHAPTER IX.

IMBEDDING METHODS: PARAFFIN AND OTHER FUSION MASSES . 79
Paraffin, 79; Soap, 94; Gelatin, 97.

CHAPTER X. PAGE

COLLODION (CELLOIDIN) AND OTHER IMBEDDING METHODS . . 99
Collodion or Celloidin, 99; other Evaporation Masses, 113; Freezing Masses, 115.

CHAPTER XI.

SERIAL SECTION MOUNTING 119
Methods for Paraffin Sections, 119; Methods for Watery Sections, 128; Methods for Celloidin Sections, 128.

CHAPTER XII.

STAINING 135

CHAPTER XIII.

CARMINE AND COCHINEAL STAINS 145
Theory of Carmine Staining, 145; Aqueous Carmines, Acid, 149; Neutral and Alkaline, 153; Alcoholic Carmines and Cochineals, 156.

CHAPTER XIV.

HÆMATEÏN (HÆMATOXYLIN) STAINS 161
Theory of Staining with Hæmatoxylin, 161; Hæmateïn (Hæmatoxylin), 163; Alumina-hæmateïn Lakes, 167; other Hæmateïn Lakes, 173.

CHAPTER XV.

ON STAINING WITH COAL-TAR COLOURS 179
Basic, Acid and Neutral Coal-tar Colours, 179; Progressive and Regressive Stains, 181; General Directions for the Regressive Method, 182.

CHAPTER XVI.

THE COAL-TAR CHROMATIN STAINS 189
Regressive Stains, 189; Progressive Stains, 197.

CHAPTER XVII.

METHYLEN BLUE 202

CHAPTER XVIII.

THE COAL-TAR PLASMA STAINS 215

CHAPTER XIX.

PAGE
OTHER ORGANIC STAINS AND COMBINATIONS 233
Other Organic Stains, 233 ; Carmine combinations, 235 ; Hæmateïn Combinations, 237.

CHAPTER XX.

METALLIC STAINS (IMPREGNATION METHODS) 241
Silver, 243 ; Gold, 250 ; other Metallic Stains, 258.

CHAPTER XXI.

EXAMINATION AND PRESERVATION MEDIA 262
Aqueous Liquids, 262 ; Mercurial Liquids, 266 ; other Fluids, 267 ; Glycerin Media, 270 ; Resinous Media, 277.

CHAPTER XXII.

CEMENTS AND VARNISHES 284

PART II

SPECIAL METHODS AND EXAMPLES.

CHAPTER XXIII.

INJECTIONS: GELATIN MASSES 293
Carmine, 296 ; Blue, 300 ; other Colours, 304.

CHAPTER XXIV.

INJECTIONS: OTHER MASSES 307
White of Egg, Gum, 307 ; Glycerin, 308 ; Aqueous, 309 ; Celloidin, 310 ; other Masses, 310.

CHAPTER XXV.

MACERATION AND DIGESTION 312
Maceration, 312 ; Digestion, 320.

CHAPTER XXVI.

CORROSION, DECALCIFICATION, AND BLEACHING . . . 322
Corrosion, 322 ; Decalcification and Desilicification, 323 ; Bleaching, 328.

CHAPTER XXVII.

PAGE

EMBRYOLOGICAL METHODS 331

CHAPTER XXVIII.

CYTOLOGICAL METHODS 354

CHAPTER XXIX.

TEGUMENTARY ORGANS 370

CHAPTER XXX.

MUSCLE AND TENDON (NERVE-ENDINGS) 378

Striated Muscle, 378; Tendon, 381; Smooth Muscle, 382.

CHAPTER XXXI.

NEUROLOGICAL METHODS.—INTRODUCTION AND SECTION METHODS . 385

CHAPTER XXXII.

NEUROLOGICAL METHODS.—NERVE-FIBRE STAINS (Weigert and others) 405

CHAPTER XXXIII.

NEUROLOGICAL METHODS.—AXIS-CYLINDER AND PROTOPLASM STAINS (Golgi and others) 415

Retina, 439; Inner Ear, 442.

CHAPTER XXXIV.

SOME OTHER HISTOLOGICAL METHODS 444

Connective Tissues, 444; Blood, 456; Glands, 461.

CHAPTER XXXV.

SOME ZOOLOGICAL METHODS 466

Tunicata, 467; Mollusca, 468; Arthropoda, 472; Vermes, 476; Echinodermata, 485; Cœlenterata, 488; Porifera, 493; Protozoa, 495.

APPENDIX 501

INDEX 507

UNIVERSITY
CALIFORNIA

THE MICROTOMIST'S VADE-MECUM.

CHAPTER I.

INTRODUCTORY.

1. **The General Method.**—The methods of modern microscopic anatomy may be roughly classed as General and Special. There is a General or Normal method, known as the method of sections, which consists in carefully *fixing* the structures to be examined, *staining* them with a *nuclear* stain, *dehydrating* with alcohol, and mounting *series of sections* of the structures in balsam. It is by this method that the work is blocked-out and very often finished. Special points are then studied, if necessary, by Special Methods, such as examination of the living tissue elements, *in situ*, or in "indifferent" media; fixation with special fixing agents; staining with special stains; dissociation by teasing or maceration; injection; impregnation; and the like.

There is a further distinction which may be made, and which may help to simplify matters. The processes of the preparation of tissues may be divided into two stages, *Preliminary Preparation* and *Ulterior Preparation.* Now the processes of preliminary preparation are essentially identical in all the methods; essential divergencies being only found in the details of ulterior preparation. By preliminary preparation is meant that group of processes called by German anatomists *Conservirungsmethoden,* those namely whose object it is to get the tissues into a fit state for passing unharmed through all the ulterior processes to which it may be desired to submit them. Preliminary preparation comprehends the operations of (1) killing; (2) fixing; (3) the washing and other manipulations necessary for removing the fixing agent from the tissues, and substituting for it the preservative liquid or other reagents which it is desired to employ.

2. Preliminary Preparation.—The first thing to be done with any structure is to *fix* its histological elements. (This statement applies equally to all classes of objects, whether it be desired to cut them into sections or to treat them in any other special way.) Two things are implied by the word "fixing;" first, the rapid *killing* of the element, so that it may not have time to change the form it had during life, but is fixed in death in the attitude it normally had during life; and second, the *hardening* of it to such a degree as may enable it to resist without further change of form the action of the reagents with which it may subsequently be treated. Too much stress can hardly be laid on this point, which is the most distinctive feature of modern histological practice; without good fixation it is impossible to get good stains, or good sections, or preparations good in any way.

The structure having been duly fixed by one of the processes described in the chapter on FIXING AGENTS, is *washed* in order to remove from the tissues as far as possible all traces of the fixing reagent.

The kind of liquid with which washing out is done is not a matter of indifference. If corrosive sublimate (for instance) or osmic acid, or a solution into which chromic acid or a chromate enters, have been used for fixing, the washing may be done with water. But if picric acid in any form has been used, the washing must be done with alcohol. The reason of this difference is that the first-named reagents (and, indeed, all the compounds of the heavy metals used for fixing) appear to enter into a state of chemical combination with the elements of tissues, rendering them insoluble in water; so that the hardening induced by these agents is not removed by subsequent treatment with water. Picric acid, on the other hand, produces only a very slight hardening of the tissues, and does not appear to enter into any combination whatever with their elements, as it is entirely removeable by treating the tissues with water or alcohol. If the removal be effected by means of water, the tissue elements are left in a soft state in which they are obnoxious to all the hurtful effects of water. Alcohol must therefore be taken to remove the picric acid and to effect the necessary hardening at the same time. Instructions for washing out are given, when necessary, in the discussion of the different fixing agents in the following parts of this work.

These operations having been duly performed, two roads become open. The object may be further prepared by what may be termed the *wet* method, in which all subsequent operations are performed by means of aqueous media. Or it may be further prepared by what may be termed the *Dehydration* method, which consists in treatment with successive alcohols of gradually increasing strength, final *Dehydration* with absolute alcohol, imbibition with an essential oil or other *clearing agent*, and lastly either mounting at once in balsam or other resinous medium or imbedding in paraffin for the purpose of making sections. The dehydration method is the course which is generally preferred. The chief reason for this lies in the great superiority of the dehydration methods as regards the preservation of tissues. The presence of water is the most important factor in the conditions that bring about the decomposition of organic matter, and its complete removal is the chief condition of permanent preservation. It is of course not intended here to suggest that wet methods of preparation should be altogether discarded. They have great value, they are even indispensable, for special ends; and all that is intended to be suggested is, that they should be regarded not as *general* but as *special* methods.

The further course of preliminary preparation by the dehydration method is as follows:

3. Dehydration and Preservation.—At the same time that the superfluous fixing agent is being removed from the tissues, or as soon as that is done, the *water of the tissues must be removed.* This is necessary for two reasons; firstly, in the interest of preservation, the presence of water being the condition of all others that most favours post-mortem decomposition; and secondly, because all water must be removed in order to allow the tissues to be impregnated with the imbedding material necessary for section-cutting, or with the balsam with which they are to be finally preserved. (The cases in which aqueous imbedding and preserving media are employed are exceptional, and will be treated of in the proper places.) The *dehydration* is performed as follows:—The objects are brought into weak alcohol, and are then passed through successive alcohols of gradually increased strength, remaining in each the time necessary for complete saturation, and the last bath consisting of absolute or at least very strong alcohol.

In dealing with extremely delicate objects, it may be necessary to take special precautions in order to avoid injury to them through the violent diffusion-currents that are set up in the passage from water to alcohol, or from one bath of alcohol to another of considerably different density. Some kind of diffusion-apparatus should be used in these cases. The objects may be placed in a tube plugged at one end and closed at the other by a diaphragm of chamois skin or other suitable membrane, the tube being then immersed in a vessel containing the grade of alcohol that it is desired to add to the liquid in the tube, and the whole allowed to remain until by diffusion through the diaphragm the two liquids have become of equal density. Or COBB's differentiator (*Proc. Linn. Soc.*, N.S.W., v, 1890, p. 157; *Journ. Roy. Mic. Soc.*, 1890, p. 821) may be employed. Or, more conveniently in most cases, the apparatus described and figured by HASWELL (*Proc. Linn. Soc.*, N.S.W., vi, 1891, p. 433; *Journ. Roy. Mic. Soc.*, 1892, p. 696). This consists of two wash-bottles connected in the usual way by tubing, and furnished, the one with an overflow-tube, and the other with a feeding-tube leading from a reservoir connected with it by means of a regulating tap or drop arrangement. The objects are placed in the first bottle; some of the same liquid as that containing the objects is placed in the second bottle; and alcohol of the grade that it is desired to add is led into it from the reservoir. The mixture of liquids therefore takes place in the bottle that does not contain the objects, and the mixture itself is gradually led over to the objects through the siphon-tube connecting the two bottles. Another apparatus for rapid dehydration, devised by CHEATLE, will be found described in *Journ. Pathol. and Bacteriol.*, i, 1892, p. 253, or *Journ. Roy. Mic. Soc.*, 1892, p. 892.

I would here call attention to the varied usefulness of the "Siebdosen" or sieve-dishes of STEINACH, ZIMMERMANN, and SUCHANNEK (vide *Zeit. f. wiss. Mik.*, iv, 4, 1887, p. 433, and vii, 2, 1890, p. 159). They consist of a covered glass capsule into which is fitted a "sieve" made of a watch-glass pierced with holes and supported on legs. It is evident that the arrangement is very handy, not only for staining, washing out, treatment with vapours, &c., but for any operation in which it is desirable to have specimens supported in the upper layers of a quantity of reagent. They are sent out in a very neat form by Dr. Grübler. FAIRCHILD's perforated porcelain cylinders for washing seem to be a very neat idea. These are made small enough to be floated by the cork that closes them. See the descriptive paper in *Zeit. f. wiss. Mik.*, xii, 3, 1896, p. 301.

It is sometimes stated that it is necessary that the last alcohol bath should consist of absolute alcohol. This however is incorrect, a strength of 90 per cent., or at all events 95 per cent., being sufficient in almost all cases. For the small amount of water that remains in the tissues after treatment with these grades of alcohol is efficiently removed in the bath of clearing agent, if a good clearing agent be employed. Oil of cedar will remove the remaining water from tissues saturated with 95 per cent. alcohol; oil of bergamot will "clear" from 90 per cent. alcohol, and anilin oil will clear from 70 per cent. alcohol.

It is not generally necessary that pure ethylic alcohol be employed for dehydration; methylated spirit will suffice in most cases.

I am not aware of any substance that can entirely take the place of alcohol for dehydration and preservation. Acetone, and methylal, have been lately (PARKER, *Zool. Anz.*, 403, 1892, p. 376) substituted for alcohol in the dehyhration of methylen-blue preparations; but the great boon of an efficient substitute for alcohol in general work remains yet to be discovered. Formaldehyde (see under Fixing and Hardening Agents) is now known to be a most admirable medium for the preservation of museum specimens, being for that purpose in many cases greatly superior to alcohol; but experience is wanting as to how far it is available for the preservation of histological material, whilst of course, occurring as it does in the form of an aqueous solution, it can have no dehydrating effect.

Considered as a mere dehydrating agent, alcohol fulfils its functions fairly well. But considered as a histological preservative agent, it is far less satisfactory. If tissues be left in alcohol for only a few days before subjecting them to the further stages of preparation, the injurious effects of a sojourn in alcohol will perhaps not be very disagreeably evident. But it is otherwise if, as must often be the case, they are put away for many weeks or months before the final preparation can be carried out. The dehydrating action of the alcohol being continuously prolonged, the minute structure of tissues is sometimes considerably altered by it; they become overhard and shrink, and become brittle, and their capacity for taking stains well becomes seriously diminished. KULTSCHITZKY (*Zeit. f. wiss. Mik.*, iv, 3, 1887, p. 349) has proposed to remedy this by putting up objects, after fixation and washing out with alcohol, in ether, xylol, or toluol. FLEMMING has lately (*Arch. f. mik. Anat.*, xxxvii, 1891, p. 685) advised putting up objects after fixation in a mixture of alcohol, glycerin, and water, in about equal parts, pointing out that objects thus preserved may be at any moment either prepared for sectioning by treatment with pure alcohol, or softened for dissection or teasing by a little soaking in water, and that they do not become so hard and brittle as alcohol specimens, and retain their staining power much better. After extensive experience of this plan, I can highly recommend it, and would only further suggest that the action of the liquid seems to me to be in many cases much improved by addition of a little acetic acid (say 0·5 to 0·75 per cent.).

For material that is intended only for section-cutting, I find that by far the best plan is to clear and imbed at once in paraffin. This affords, as far as I can see, an absolutely perfect preservation. I have been working lately on some material that has been preserved in this way for over seven years. The preservation of the tissues, down to the finest details of cell-structure, appears to be perfect, and the staining, though perhaps somewhat slowed, is as precise as when the specimens were first put up, or more so. The only noticeable defect is that the tissues are rather brittle, and do not cut well; but it is not certain that that is not owing to their having been over-hardened in the first instance. Cedar-wood oil is, I find, nearly, if not quite, as good as paraffin.

4. Ulterior Preparation. Clearing.—The water having been thus sufficiently removed, the dehydration method proceeds as follows:—The alcohol is in its turn removed from the tissues, and its place taken by some anhydrous substance, generally an essential oil, which is miscible with the material used for imbedding. This operation is known as *Clearing*. It is very important that the passage from the last alcohol to the clearing agent be made gradual. This is effected by placing the clearing medium *under* the alcohol. A sufficient quantity of alcohol is placed in a tube (a watch-glass will do, but tubes are generally better), and then with a pipette a sufficient quantity of clearing medium is introduced *at the bottom of the alcohol*. Or you may first put the clearing medium into the tube, and then carefully pour the alcohol on to the top of it. The two fluids mingle but slowly. The objects to be cleared being now quietly put into the supernatant alcohol, float at the surface of separation of the two fluids, the exchange of fluids takes place gradually, and the objects slowly sink down into the lower layer. When they have sunk to the bottom, the alcohol may be drawn off with a pipette, and the objects will be found to be completely penetrated by the clearing medium. (It may be noted here that this method of making the passage from one fluid to another applies to all cases in which objects have to be transferred from a lighter to a denser fluid—for instance, from alcohol, or from water to glycerine. It is a *more exact* method than that of successive baths of mixture of alcohol and clearing agent.)

It should also be noted here that this is the proper stage for carrying out minute dissections, if any such have to be done, a drop of clearing medium agent being a most helpful medium for carrying out such dissections in, as will be further explained later on (§ 8).

5. Imbedding, and Treatment of Sections.—The objects are now *imbedded*. They are removed from the clearing medium, and soaked until thoroughly saturated in the imbedding medium. This is, for small objects, generally paraffin, liquefied by heat, and for large objects generally a solution of collodion or "celloïdin" (in this latter case the clearing is omitted, the tissues being imbedded direct from the alcohol). The imbedding medium containing the object is then made to solidify, as described in the chapter on imbedding processes, and sections are made with a microtome through the imbedding mass and the included objects. The sections are then mounted on a slide by one of the methods described in the chapter on Serial Section Methods, the imbedding material is removed from them (in the case of paraffin), they are stained *in situ* on the slide, dehydrated with alcohol, cleared and mounted in balsam or damar. Or they may be stained, washed, dehydrated, and cleared in watch-glasses, and afterwards mounted as desired—the imbedding medium being first removed if desirable.

It is not always desirable to remove the imbedding mass; celloidin sections stain well without being freed from it, and are usually even dehydrated, cleared, and mounted without removal of the mass, which becomes quite transparent in balsam. This plan has the advantage, which is a very important one for large sections, of allowing the sections to remain during the whole of the manipulations protected by a supporting mass that holds all their parts together.

The plan of staining sections on the side is of somewhat recent introduction; before it had been worked out the practice was to stain structures *in toto*, before cutting sections. In this case the object, after having been fixed, and washed out, is taken from the water, or while still on its way through the lower alcohols (it should not be allowed to proceed to the higher grades of alcohol before staining, if that can be avoided), and passed through a bath of stain (generally alcoholic borax-carmine or other alcoholic stain) of sufficient duration, then dehydrated with successive alcohols, passed through a clearing medium into paraffin, cut, and treated as above described, the sections in this case being mounted direct from the turpentine, naphtha, or other solvent with which the paraffin is removed. If aqueous staining media be applied (and it is sometimes very desirable for particular purposes to prepare specimens with some aqueous stain) the structures should either be stained *in toto* immediately after fixing and washing out, or sections may be stained on the slide, the objects being passed through successive baths of alcohol of gradually decreasing strength before being put into the aqueous stain (a precaution which will not be necessary for chromic objects (*see* below, § 9)).

But it is generally advisable not to stain in bulk material that is intended to be sectioned; much time is saved by staining it as sections, with the further advantage that the staining can be much better controlled, and that many excellent stains can in this way be employed that are not available for staining in bulk.

6. Résumé of the Section-Method.—It was stated in the first edition of this work that " the great majority of preparations are made by fixing either with sublimate or a picric acid combination, washing out with alcohol, staining with alcoholic borax-carmine, imbedding in chloroform paraffin, cutting with a sliding microtome, and mounting the sections in series in Canada balsam." But histological practice has greatly changed since then, and I would now suggest the following: —Fix either in sublimate, or preferably in many cases in Flemming's chromo-aceto-osmic mixture, or one of the other fixing agents recommended in later chapters; wash out; dehydrate; clear with oil of cedar-wood; imbed in paraffin; mount sections on the slide with Mayer's albumen medium; stain as desired; and mount in balsam or damar. That, or something like that, is now the practice of many of the most advanced workers; and I know of no method that seems to have equal claims to be considered a classical method of general morphological investigation.

As regards the method to be used for imbedding, I take it that the paraffin method is the method *par excellence* for small objects (objects up to 5 or 7 millimetres diameter); whilst the collodion or "celloïdin" method is the method *par excellence* for large objects.

As above said, I consider the practice of staining section material in bulk to be superannuated.

7. Preparation of Entire Objects, or of Material that is not to be Sectioned.—The treatment of objects which can be studied without being cut into sections is identical with that above described, with the omission of those passages that relate to imbedding processes. Its normal course may be described as fixation, washing out, staining, treatment with successive alcohols of gradually increasing strength, final dehydration with absolute alcohol, clearing, and mounting in balsam. This method is usually preferred, as a general method, to the wet methods, for the reasons that have been given above (§ 2), and for some others, amongst which may be noted the greater transparency given to tissues by mounting them in media of high refractive index, such as balsam.

In the *preparation of entire objects* or structures that are intact and *covered by an integument not easily permeable* by liquids, special care must be taken to avoid swelling from endosmosis on the passage of the objects from any of the liquids employed to a liquid of less density, or shrinkage from exosmosis on the passage to a liquid of greater density. This applies most specially to the passage from the last alcohol into the clearing medium. A slit should be made in the integument, if possible, so that the two fluids may mingle without hindrance. And in all cases the passage is made gradual by placing the clearing medium under the alcohol, as above described. Fluids of high diffusibility should be employed as far as possible in all the processes. Fixing agents of great penetrating power (such as picrosulphuric acid or alcoholic sublimate solution) should be employed where the objects present a not easily permeable integument. Washing out is done with successive alcohols, water being used only in the case of fixation by osmic acid, or the chromic mixtures or other fixing solutions that render washing by water imperative. Staining is done by preference with alcoholic staining media. The stains most to be recommended are Grenacher's borax-carmine, or one of Mayer's new carminic acid or hæmatein stains (for all of which *see* STAINING AGENTS). Anilin stains are rarely applicable to this class of preparations. Aqueous stains are more seldom used, though there are many cases in which they are admissible, and some in which they are preferable.

8. Minute Dissections.—These are best done, if necessary, in a drop of clearing agent. I recommend cedar-wood oil for this purpose, as it gives to the tissues a consistency very favorable for dissection, whilst its viscosity serves to lend support to delicate structures. Clove oil has a tendency to make tissues that have lain in it for some time very brittle. The brittleness is, however, sometimes very helpful in minute dissections. Another property of clove oil is that it does not easily spread itself over the surface of a slide, but has a tendency to form very convex drops. This property also makes it frequently a very convenient medium for making minute dissections in.

REESE
UNIV
CALIFORNIA

9. General Principles.—Following Paul Mayer, I gave in the previous editions the following reasons for employing for entire objects alcoholic rather than aqueous staining media. Since, in most cases, treatment with alcohol forms part of the fixing process, alcoholic solutions are logically indicated for staining. For by means of them it is possible to avoid the bad effects that follow on passing delicate tissues from alcohol into water, violent diffusive currents being thereby set up which sometimes carry away whole groups of cells; swellings being caused in the elements of the tissues; and, if the immersion in the aqueous medium be prolonged, as is generally necessary in order to obtain a thorough stain, maceration of the tissues supervening. But alcoholic staining fluids have still other advantages; they are in general more penetrating; with them alone is it possible to stain through chitinous integuments; and, if it be desired to stain slowly, tissues may be left in them for days without hurt.

Applied to the case now under consideration, the preparation *in toto* of objects protected by not easily permeable investments, this doctrine is evidently a wise one. For such objects must necessarily be fixed by some highly penetrating but not permanently hardening agent such as picric acid, and must necessarily be washed out with alcohol; and it is a good maxim for tissues so fixed that an object that has once been in alcohol should not be allowed to go back into water, if that can possibly be avoided.

But in the case of structures that have been well fixed in a strongly and permanently coagulating medium such as chromic acid, this precaution is much less necessary. Sections of tissues that have been fixed for twenty-four hours in Flemming's solution may be passed with relative impunity from absolute alcohol into an aqueous stain, and from that back again direct into absolute alcohol. It is this property of tissues fixed in chromic solution that determines me to recommend the practice of staining sections, instead of staining objects *in toto*. As regards the *quality* of the stain, aqueous solutions are in general the best. No alcoholic carmine or hæmatoxylin, for instance, will give a stain equal in precision and delicacy to those given by alum-carmine or Böhmer's hematoxylin. In a recent publication Mayer himself states that he has somewhat cooled in his preference for alcoholic staining media.

For an excellent exposition of the principles underlying the practice above recommended, the reader may consult with advantage the paper of Paul Mayer, in *Mitth. Zool. Stat. Neapel*, ii (1881), p. 1, *et seq*. See also the abstract in *Journ. Roy. Mic. Soc.* (N.S), ii (1882), pp. 866—881, and that in *Amer. Natural.*, xvi (1882), pp. 697—706, in which two last some improvements are mentioned which have been worked out since the publication of Mayer's paper.

CHAPTER II.

KILLING.

10. In the majority of cases, the first step in the preparation of an organ or organism consists in exposing it as rapidly and as completely as possible to the action of one of the **Fixing Agents** that are discussed in the next chapter. The organ or organism is thus taken in the normal living state; the fixing agent serves to bring about at the same time, and with sufficient rapidity, both the death of the organism and that of its histological elements.

But this method is by no means applicable to all cases. There are many animals, especially such as are of a soft consistence, and deprived of any rigid skeleton, but possessing a considerable faculty of contractility—such as many Cœlenterata, Bryozoa, and Serpulida, for instance—which if thus treated contract violently, draw in their tentacles or branchiæ, and die in a state of contraction that renders the preserved object a mere caricature of the living animal. In these cases, special methods of killing must be resorted to.

Sudden Killing.

11. Heat.—Speaking generally, there are two ways of dealing with these difficult cases. You may kill the animal so suddenly that it has not time to contract; or you may paralyse it by narcotics before killing it.

The application of *Heat* is a good means of killing suddenly. It has the great advantage of allowing of good staining subsequently, and of hindering less than any other method the application of chemical tests to the tissues. By it the tissues are fixed at the same time that somatic death is brought about.

The difficulty consists in hitting off the right temperature, which is of course different for different objects. I think that a temperature of 80° to 90° C. will generally be amply sufficient, and that very frequently it will not be necessary to go beyond 60° C. An exposure to heat for a few seconds will generally suffice.

Small objects (Protozoa, Hydroids, Bryozoa) may be brought into a drop of water in a watch-glass or on a slide and heated over the flame of a spirit lamp. For large objects, the water or other liquid employed as the vehicle of the heat may be heated beforehand and the animals thrown into it.

As soon as it is supposed that the protoplasm of the tissues is coagulated throughout, the animals should be brought into alcohol (30 to 70 per cent. alcohol) (if water be employed as the heating agent).

An excellent plan for preparing many marine animals is to kill them in *hot fresh* water. Some of the larger Nemertians are better preserved by this method than by any other with which I am acquainted.

12. Slowly Contracting Animals.—Animals that contract but slowly, such as *Alcyonium* and *Veretillum,* and some Tunicates, such as *Pyrosoma,* are very well killed by throwing them into some very quickly acting fixing liquid, either used hot or cold. *Glacial* or very strong *acetic acid* (VAN BENEDEN'S method) is an excellent reagent for this purpose; it may be used, for example, with some Medusæ. After an immersion of a few seconds or a few minutes, according to the size of the animals, they should be brought into alcohol of at least 50 per cent. strength. See "**Acetic acid**" and "**Tunicata.**" *Lemon juice* employed in this way has given me very good results with small Annelids and Hirudinea. *Corrosive sublimate* is another excellent reagent for this purpose.

Narcotisation.

13. The secret of narcotisation consists in adding some anæsthetic substance very gradually, in very small doses, to the water containing the animals, and waiting patiently for it to take effect slowly.

THE TOBACCO-SMOKE METHOD for Actiniæ (HERTWIG, *Die Actinien,* 1879) used to be practised as follows:—A dish containing the animals in water is covered with a bell-glass, under which passes a curved glass or rubber tube, which dips into the water. Tobacco-smoke is blown into the water for some time, through the tube, and the animals are then left for some hours in order that narcotisation may become fully established. The animals are irritated from time to time by touching a tentacle with a needle.

As soon as it is observed that an animal begins to react slowly, that is to say as soon as it is found that the contraction of the tentacle does not begin until a considerable time after it has been irritated by the needle, the narcotisation may be considered sufficient. A quantity of some fixing liquid sufficient to kill the animals before they have time to contract is then added to the water.

A space of several hours is necessary in order to thoroughly narcotise an Actinia by this method.

14. **Nicotin in solution** may be used instead of tobacco smoke (ANDRES, *Atti R. Accad. dei Lincei*, v, 1880, p. 9; see *Journ. Roy. Mic. Soc.*, N.S., ii, 1882, p. 881). Andres employs a solution of 1 gramme of nicotin in a litre of sea-water. The animal to be anæsthetised is placed in a jar containing half a litre of sea-water, and the solution of nicotin is gradually conducted into the jar by means of a thread acting as a syphon. The thread ought to be of such a thickness as to be capable of carrying over the whole of the solution of nicotin in twenty-four hours.

15. **Chloroform** may be employed either in the liquid state or in the state of vapour. KOROTNEFF (*Mitth. Zool. Stat. Neapel*, v, Hft. 2, 1884, p. 229; *Zeit. f. wiss. Mik.*, 2, 1885, p. 230) operates in the following manner with Siphonophora. The animals being extended, a watch-glass containing chloroform is floated on the surface of the water in which they are contained, and the whole is covered with a bell-glass. As soon as the animals have become insensible they are killed by means of hot sublimate or chromic-acid solution plentifully poured on to them.

Liquid chloroform is employed by squirting it in small quantities on to the surface of the water containing the animals. A syringe or pipette having a very small orifice, so as to thoroughly pulverise the chloroform, should be employed. Small quantities only should be projected at a time, and the dose should be repeated every five minutes, until the animals are anæsthetised.

I have seen large Medusæ very completely anæsthetised in the state of extension in an hour or two by this method. ANDRES finds that this plan does not succeed with Actiniæ, as with them maceration of the tissues supervenes before anæsthesia is established.

16. Ether and **Alcohol** may be administered in the same way. ANDRES has obtained good results with Actiniæ by the use of a mixture (invented by SALVATORE LO BLANCO) containing 20 parts of glycerin, 40 parts of 70 per cent. alcohol, and 40 parts of sea-water. This mixture should be carefully poured on to the surface of the water containing the animals, and allowed to diffuse quietly through it. Several hours are sometimes necessary for this.

EISIG employs alcohol in the same way.

17. Methyl-alcohol.—CORI (*Zeit. f. wiss. Mik.*, vi, 4, 1890, p. 438) prefers methyl-alcohol to all other reagents. It has, amongst other advantages, that of having but a slight action on albumins. CORI recommends a mixture composed of 10 c.c. methyl-alcohol (of 96 per cent. strength), 90 c.c. water (fresh or sea-water), and 0·6 g. of sodium chloride (to be added only when fresh water is taken, the addition of the salt having for its object to prevent maceration). It may be well to add to this mixture a very few drops of chloroform (for *Cristatella*; *Zeit. f. wiss. Zool.*, lv, 1893, p. 626; *Zeit. f. wiss. Mik.*, x, 4, 1893, p. 475).

18. Hydrate of Chloral, which was first recommended, I believe, by Foettinger (*Arch. de Biol.*, vi, 1885, p. 115), gives very good results with some subjects. Foettinger operates by dropping crystals of chloral into the water containing the animals. For Alcyonella he takes 25 to 80 centigrammes of chloral for each hundred grammes of water. It takes about three quarters of an hour to render a colony sufficiently insensible to allow of fixing. Foettinger has obtained satisfactory results with marine and fresh-water Bryozoa, with Annelida, Mollusca, Nemertians, Actiniæ, and with *Asteracanthion*. He did not succeed with Hydroids.

I am bound to state that I have never had the slightest success with Nemertians.

VERWORN (*Zeit. f. wiss. Zool.*, xlvi, 9, 1887, p. 99; see also *Journ. Roy. Mic. Soc.*, 1888, p. 148) operates differently for fresh-water Bryozoa. He puts *Cristatella* for a few minutes into 10 per cent. solution of chloral, in which the animals sooner or later become extended.

KÜKENTHAL (*Zeit. f. wiss. Mik.*, iv, 3, 1887, p. 378; *Journ. Roy. Mic. Soc.*, 1888, p. 509) has obtained good results with some Annelids, by means of a solution of one part of chloral in 1000 parts of sea-water.

The chloral method gives rise to maceration with some subjects, and has been found to distort nuclear figures. (This, I suspect, will probably be found to be the case with most of these methods.)

19. **Cocaine** (Richard; *Zool. Anz.*, 196, 1885, p. 332) has been found to give good results. Richards puts a colony of Bryozoa into a watch glass with 5 c.c. of water, and adds gradually 1 per cent. solution of hydrochlorate of cocaine in water. After five minutes, the animals are somewhat numbed, and half a cubic centimetre of the solution is added, and the tentacles are caused to contract by irritating them with a needle. Ten minutes later the animal should be found to be dead in a state of extension.

This method is stated to succeed with Bryozoa, *Hydra*, and certain worms. It is the best method for Rotifers (Rousselet).

It has been pointed out (by Cori, in the paper quoted above) that unfortunately when fixing agents, such as sublimate solution, are added to the animals, the cocaine is thrown down on them as a white precipitate.

20. **Hydroxylamin.**—Hofer (*Zeit. f. wiss. Mik.*, vii, 3, 1890, p. 318) has employed hydroxylamin as a paralysing agent with success with the most varied animal forms. Either the sulphate or the hydrochlorate of the base may be used. He recommends that the hydrochlorate be taken. This, as found in commerce, is usually contaminated with HCl. It should be dissolved in water (spring or sea-water, according to the habitat of the organisms—in no case distilled water) and the solution exactly neutralised by addition of carbonate of soda. A 1 per cent. solution should be made up, and further diluted for use. The organisms are placed in the diluted solution, which may be taken of a strength varying from 0·1 per cent., used for thirty minutes or less (as for Infusoria), to 0·25 per cent., used for from fifteen minutes to one hour (*Hydra*), 1 per cent., one half to two hours (*Hirudo*), or as much as ten to twenty hours (*Helix* and *Anodonta*).

It should be remembered that hydroxylamin is an extremely powerful reducing agent. Care must therefore be taken not to treat the paralysed animals with easily reducible fixing agents, such as osmic acid, chromic acid, sublimate, chlorides of gold or platinum, &c., unless it have been possible first to sufficiently wash out the hydroxylamin with water.

21. Chloride of Magnesium.—TULLBERG (*Arch. Zool. Expér. et Gén.*, x, 1892, p. 11; *Journ. Roy. Mic. Soc.*, 1892, p. 435) has obtained some results with this salt. For Actiniæ, a 33 per cent. solution of the salt is to be very slowly added to the water containing the expanded animal, until the vessel contains 1 per cent. of the salt (thus for one litre of sea-water 33 c.c. of the solution must be added). The addition must be made gradually; but it must be effected within half an hour. Thirty minutes later the animal will be found to be anæsthetised, and may be fixed.

For terrestrial and fresh-water Invertebrates, rather stronger solutions should be used.

REDENBAUGH (*Amer. Natural.*, xxix, 1895, p. 399; *Journ. Roy. Mic. Soc.*, 1895, p. 385) has obtained good results by means of **Sulphate of Magnesium**, either added in crystals to the sea-water containing the animals, until a saturated solution is obtained; or in the shape of a saturated solution into which they are thrown (Annelids).

22. Poisoning by small doses of some fixing agent is sometimes a good method. SALVATORE LO BLANCO employs the following method for preserving Ascidiæ in an extended state. A 1 per cent. solution of chromic acid acidulated with acetic acid is poured on to the surface of the water containing the animals, and allowed to diffuse slowly through it. The operation takes four or five days (v. GARBINI, *Manuale per la Technica Mod. del Microscopio*, p. 168).

Osmic acid, or Kleinenberg's solution, is sometimes employed in the same way.

I have seen Medusæ killed in a satisfactory manner by means of crystals of corrosive sublimate added to the water containing them.

Morphia, Curare, Strychnin, Prussic Acid, and other paralysing drug have also been employed.

23. Asphyxiation may be sometimes successfully practised. Terrestrial Gastropods may be killed for dissection by putting them into a jar quite full of water that has been deprived of its air by boiling, and hermetically closed. After from twelve to twenty-four hours the animals are generally found dead and extended. The effect is obtained somewhat quicker if a little tobacco be added to the water.

Good results are sometimes obtained with aquatic animals by simply leaving them to exhaust the oxygen of the water in which they are contained. I have sometimes succeeded with

Holothuriæ and other Echinoderms in this way; and WARD (see *Amer. Nat.*, xxv, 1891, p. 398) has succeeded with Hydroids, Actiniæ, and similar forms. If the animals be found to be imperfectly expanded when narcosis has set in, they may be got to expand by putting them back for a short time into pure sea-water; and as soon as they are expanded should be quickly thrown into some rapidly-killing reagent.

24. **Carbonic Acid Gas** has been recommended (by FOL, *Zool. Anz.*, 128, 1885, p. 698). The water containing the animals should be saturated with the gas. The method is stated to succeed with most Cœlenterata and Echinodermata, but not with Molluscs or Fishes. It has not been found successful at the Naples Zoological Station. I have had most excellent results with small Annelids and Hirudinea. It is not necessary to employ a generator for obtaining the gas. It suffices to take an ordinary "soda-water" siphon, and squirt its contents into the water containing the animals. Narcotised animals recover very quickly on being put back into pure water.

25. **Marine Animals** are sometimes successfully killed by simply putting them into fresh water.

Warm Water will sometimes serve to immobilise and even kill both marine and fresh-water organisms.

REESE LIBRARY OF THE UNIVERSITY OF CALIFORNIA

CHAPTER III.

FIXING AND HARDENING.

26. The Necessity of Fixing.—The meaning of the term "fixing" has been explained above (§ 2). It only remains here to insist on the absolute necessity of the employment of fixing agents, and to briefly illustrate this necessity by a single example. If a portion of living retina be placed in aqueous humour, serum, or other so-called "indifferent" medium, or in any of the media used for permanent preservation, it will be found that the rods and cones will not preserve the appearance they have during life for more than a very short time; after a few minutes a series of changes begins to take place, by which the outer segments of both rods and cones become split into discs, and finally disintegrate so as to be altogether unrecognisable, even if not totally destroyed. Further, in an equally short time the nerve-fibres become varicose, and appear to be thickly studded with spindle-shaped knots; and other post-mortem changes rapidly occur. If, however, a fresh piece of retina be treated with a strong solution of osmic acid, the whole of the rods and cones will be found perfectly preserved after twenty-four hours' time, and the nerve-fibres will be found not to be varicose. After this preliminary hardening, portions of the retina may be treated with water (which would be ruinous to the structures of a fresh retina), they may even remain in water for days without harm; they may be stained, acidified, hardened, imbedded, cut into sections, and mounted in either aqueous or resinous media without suffering.

27. The Action of Fixing Agents consists in the coagulation of certain of the constituents of tissues, of their albuminoids, their gelatin, their mucin. Some fixing agents seem to have further the property of combining chemically with the tissues, so that they cannot be easily removed from them by washing. This is a consideration of great importance in view of ulterior operations, and most particularly in view of staining. Chromic acid and its salts, osmic acid, the chlorides of palladium, of gold, and of iron, are reagents that seem to combine chemically with the tissues, and render necessary a special after-treatment and special modes of staining, whilst picric acid, nitric acid, and corrosive sublimate do not appear to enter into that kind of combination, and can be entirely removed from the tissues by washing, and leave the tissues in a state in which they are susceptible of any kind of staining.

Practically it amounts to this, that if you fix with a chromic or osmic mixture, you cannot stain with carmine, but can only stain with hæmatoxylin, if you wish to stain your objects *in toto;* or you may make sections, and stain them with safranin or some other coal-tar colour. Whilst if you fix with sublimate or a picric acid mixture, you may do as you like in the matter of staining.

The after-treatment appropriate to each fixing agent is indicated in the special paragraphs.

28. Choice of a Fixing Agent.—Indications concerning the proper fixing agent to employ for the different tissues and organs of the animal kingdom will be found in Part II. The following remarks are intended as hints for beginners only.

The chief fixing agents for general work are FLEMMING'S *mixture,* HERMANN'S *platinum-chloride mixture,* corrosive sublimate, and PERENYI'S mixture.

I recommend that Flemming's mixture should be used wherever it is possible, as I believe it to be in general by far the best fixing agent yet invented, with the exception of Hermann's mixture, which is unfortunately too expensive to be employed in large quantities.

But it will not always be found possible to use it. Its low power of penetration, for instance, puts it out of court in the case of very impermeable objects, such as are frequently offered by the Arthropoda. For these, picro-sulphuric acid may be recommended.

For very small objects, such as may be mounted whole, osmic acid is nearly as good as Flemming's mixture, and is frequently much more convenient to use.

For objects a little larger, and for much embryological work on objects that it is convenient to have stained *in toto,* corrosive sublimate may be recommended. I think it is in general a much better preservative of the forms of anatomical elements than either Altmann's nitric acid or Kleinenberg's fluid.

29. The Practice of Fixation.—Hints and Cautions.—See that the structures are *perfectly living* at the instant of fixation, otherwise you will only fix pathological states or post-mortem states.

Do all you can to facilitate the *rapid penetration* of the fixing agent. To this end, let the structures be divided into the smallest portions that can conveniently be employed, and if entire organs or organisms are to be fixed whole, let openings, as large as possible, be first made in them.

The penetration of reagents is greatly facilitated by *heat.* You may warm the reagent and put it with the objects to be fixed in the paraffin stove, or you may even employ a fixing agent heated to boiling point (as boiling sublimate solution for certain corals and Hydroids, or boiling absolute alcohol for certain Arthropods with very resistant integuments).

Let the *quantity* of fixing agent employed be at least *many times* the volume of the objects to be fixed. If this precaution be not observed the composition of the fixing liquid may be seriously altered by admixture of the liquids or of the soluble substances of the tissues thrown into it. For a weak and slowly acting fixing agent, such as picric acid, the quantity of liquid employed should be in volume about one hundred times that of the object to be fixed. Reagents that act very energetically, such as Flemming's solution, may be employed in smaller proportions.

Be careful to use the *appropriate liquid for washing out* the fixing agent after fixation. It is frequently by no means a matter of indifference whether water or alcohol be employed for washing out. Sometimes water will undo the whole work of fixation (as with picric acid). Sometimes alcohol causes precipitates that may ruin the preparations. Instructions on this head are given in the paragraphs devoted to the different fixing agents.

Use *liberal quantities* of liquid for washing.

Change the liquid as often as it becomes turbid, if that should happen.

The process of washing out is often greatly facilitated by *heat*. Picric acid, for instance, is nearly twice as soluble in alcohol warmed to 40° C. as in alcohol at the normal temperature (Fol).

30. Fixation of Marine Animals.—In the case of *marine organisms* it may be stated as a general rule that their tissues are more refractory to the action of reagents than are the tissues of corresponding fresh-water or terrestrial forms, and fixing solutions should in consequence be stronger (about two to three times stronger, according to Langerhans).

Marine animals ought to be *freed from the sea-water* adherent to their surface before treating them either with alcohol or any fixing reagent that precipitates the salts of sea-water. If this be not done, the precipitated salts will form on the surfaces of the organisms a crust that prevents the penetration of reagents to the interior, thus allowing maceration to be set up, and hindering the penetration of staining fluids. Fixing solutions for marine organisms should therefore be such as serve to keep in a state of solution, and finally remove, the salts in question. They should *never be made with sea-water* as a menstruum, as some workers have inconsiderately proposed. If alcohol be employed it should be *acidified* with hydrochloric or some other appropriate acid. Picro-sulphuric acid is a reagent that fulfils the conditions here spoken of. (On this subject see Paul Mayer, in *Mitth. Zool. Stat. Neapel*, ii (1881), p. 1 et seq. See also the abstract in *Journ. Roy. Mic. Soc.* (N.S.), ii (1882), pp. 866—881, and that in *Amer. Natural.*, xvi (1882), pp. 697—706.)

31. The Obligation of Hardening.—Methods of imbedding have now been brought to such a degree of perfection that the thorough hardening of soft tissues that was formerly necessary in order to cut thin sections from them is now, in the majority of cases, no longer necessary; by careful infiltration with paraffin or some other good infiltration mass, most soft objects can be satisfactorily cut with no greater an amount of previous hardening than is furnished by the usual passing of the tissues after fixing through successive alcohols in order to prepare them for the paraffin bath. But there are some exceptions. Such are, for instance, the cases in which it is desired to cut very large sections, such as sections of the entire human brain. Such an organ as this cannot be duly infiltrated with alcohol in a few hours, and it is doubtful whether it can be duly infiltrated with paraffin or any other imbedding mass in any reasonable time. And certain organs that are either extremely delicate or inaccessible, such as retina or cochlea, will require to be specially hardened in order to give the best results. The processes employed for hardening such specimens as these will be described when treating of the organs in question. In this chapter I confine myself to such general statements concerning the employment of the usual hardening agents as appear likely to be generally useful.

32. The Practice of Hardening—Hints and Cautions.—Employ *in general* a *relatively large volume* of hardening liquid, and change it very frequently. The exact proportions may be made out by experiment for each reagent and each class of objects. If the volume of liquid be insufficient its composition will soon become seriously altered by the diffusion into it of the soluble substances of the tissues; and the result may be a macerating instead of a hardening liquid. Further, as soon as, in consequence of this diffusion, the liquid has acquired a composition similar in respect of the proportions of colloids and crystalloids contained in it to that of the liquids of the tissues, osmotic equilibrium will become established, and diffusion will cease. That is to say, the hardening liquid will cease to penetrate. This means, of course, maceration of internal parts. On the other hand, it appears that a certain slight proportion of colloids in the hardening liquid is favorable to the desired reaction, as it gives a better consistency to the tissues by preventing them from becoming brittle. Hence the utility of employing *a certain proportion* of hardening agent.

Hardening had better be done in tall cylindrical vessels, the objects being suspended by a thread at the top of the liquid. This has the advantage of allowing diffusion to take place as freely as possible, whilst any precipitates that may form fall harmlessly to the bottom.

Always *begin* hardening with a *weak reagent,* increasing the strength gradually, as fast as the tissues acquire a consistence that enables them to support a more energetic action of the reagent.

Let the objects be removed from the hardening fluid as soon as they have acquired the desired consistency.

As to the *choice of a hardening reagent,* if you wish, above all, for a rapid and energetic action, take chromic acid. If you wish for a more moderate and more equable action, take a chromic salt, or one of the compounds of which the chromic salts are the chief ingredients, or one of the platinum chloride mixtures.

CHAPTER IV.

FIXING AGENTS. MINERAL ACIDS AND THEIR SALTS.

33. Osmic Acid.—The tetroxide of osmium (OsO_4) is the substance commonly known as osmic acid, though it does not possess acid properties. It is a substance that is exceedingly difficult to keep in use for any length of time. It is extremely volatile, and in the form of an aqueous solution becomes partially reduced with great readiness in presence of the slightest contaminating particle of organic matter. (It is generally believed that the aqueous solutions are reduced by light alone, but this is not the case: they may be exposed to the light with impunity if dust be absolutely denied access to them. It would even seem that the solutions are improved for some purposes by exposure to sunlight, *vide infra*, the remarks on gold chloride solutions. (Some observations communicated to me by Dr. Lindsay-Johnson go to prove that, if dust be avoided, solutions keep better in the light, with occasional "sunning," than in the dark.)

Great stress is laid by authors on the fact that the vapour of osmium is very irritating to mucous tissues. It is said that the slightest exposure to it is sufficient to give rise to serious catarrh, irritation of the bronchial tubes, laryngeal catarrh, conjunctivitis, &c. I have never myself suffered in this way, but there is no doubt that many persons do, and such susceptible subjects should be very careful in handling osmium in any form.

34. How to keep the Solutions.—After having carefully tried several of the plans that have been recommended for keeping the working solutions free from dust, I have come to the conclusion that the following is the most practical plan for preventing them from "going bad":—The solution of osmic acid in chromic acid solution is not, like the solution in pure water, easily reducible, but may be kept without any special precautions. I therefore keep the bulk of my osmium in the shape of a 2 per cent. solution of osmic acid in 1 per cent.

aqueous chromic acid solution. This solution serves for fixation by osmium vapours, and for making up solution of Flemming (or solution of Hermann) which is the form in which osmium is most generally employed. A small quantity of osmic acid may also be made up in 1 per cent. solution in distilled water, and kept carefully protected from dust for use in special cases. Those who have to do a great deal of fixing by means of the vapours may also keep a supply of the solid oxide for this purpose.

Grübler and Co. now send out osmic acid in tubes containing *one tenth* of a gramme.

Cori (*Zeit. f. wiss. Mik.*, vi, 4, 1890, p. 442) finds that solutions in distilled water keep perfectly if there be added to them enough permanganate of potassium to give a very slight rosy tint to the liquid. From time to time, as the solution becomes colourless, further small quantities of the salt should be added, so as to keep up the rosy tint.

35. Regeneration of reduced Solutions.—Bristol (*Amer. Nat.*, xxvii, 1893, p. 175; *Journ. Roy. Mic. Soc.*, 1893, p. 564; *Ref. Handbook Med. Sci.*, *Supp.*, p. 442) says that reduced solutions may be regenerated by oxidising them by means of peroxide of hydrogen. The reaction is stated to be identical with that which takes place in the bleaching of osmium-blackened tissues by peroxide. It is admitted that the tetroxide of osmium, OsO_4, is reduced by contact with organic matter into the deutoxide, OsO_2. Then, $OsO_2 + 2H_2O_2 = OsO_4 + 2H_2O$.

According to Bristol, for regenerating 100 c.c. of 1 per cent. solution of osmic acid (*erratim* 10 per cent in *Journ. Roy. Mic. Soc.*), ten to twenty drops of fresh peroxide solution should be added.

Kolossow (*Zeit. f. wiss. Mik.*, ix, 1, 1892, p. 40) says that half-reduced solutions, so long as they have not lost their characteristic odour, may be regenerated by the addition of a little powdered potash-alum.

36. Fixation by the Vapours.—Osmic acid is frequently employed in the form of vapour, and its employment in this form is indicated in most of the cases in which it is possible to expose the tissues directly to the action of the vapour. The tissues are pinned out on a cork which must fit well into a wide-mouthed bottle in which is contained a little solid osmic acid (or a small quantity of 1 per cent. solution will do). Very small objects, such as isolated cells, are simply placed on a slide, which is inverted over the mouth of the bottle. They remain there until they begin to turn brown (isolated cells will generally be found to be sufficiently fixed in thirty seconds, whilst in order to fix the deeper layers of relatively thick objects, such as retina, an exposure of several hours may be desirable). It is well to wash the objects with water before staining, but a very slight washing will suffice. For staining, methyl-green may be recommended for objects destined for study in an aqueous medium, and, for permanent preparations, alum-carmine, picro-carmine, or hæmatoxylin.

In researches on nuclei, it is possible and may be useful to employ the vapours of a freshly-prepared mixture of osmic and formic or acetic acid (Gilson, *La Cellule*, i, 1885, p. 96).

The reasons for preferring the process of fixation by vapour of osmium, where practicable, are that osmium is more highly penetrating when employed in this shape than when employed in solution, and produces a more *equal* fixation, and that the arduous washing out required by the solutions is here done away with. In many cases delicate structures are better preserved, all possibility of deformation through osmosis being here eliminated.

37. Fixation by Solutions.—When employed in aqueous solutions osmic acid is used in strengths varying from $\frac{1}{20}$ per cent. to 2 per cent. Solutions of $\frac{1}{2}$ per cent. to 1 per cent. have been very largely used, but the tendency of modern practice seems to be towards weaker solutions and longer immersion. For Infusoria $\frac{1}{2}$ per cent. for a few seconds; for Porifera $\frac{1}{20}$ to $\frac{1}{10}$ per cent. for some hours; for Mollusca 1 to 2 per cent. for twenty-four hours; for epithelia $\frac{1}{10}$ to $\frac{1}{2}$ per cent. for an hour or two; for meroblastic ova $\frac{1}{10}$ per cent. for twenty-four hours; for medullated nerve-fibre $\frac{1}{10}$ to 1 per cent. for from twenty minutes to two hours; for tactile corpuscles $\frac{1}{3}$ to 1 per cent. for twenty-four hours; for retina $\frac{1}{4}$ to 2 per cent. for from ten minutes to twenty-four hours; for nuclei $\frac{1}{10}$ to 2 per cent. for two or three hours. Such figures as these will serve to give a general idea of the practice, whilst more precise instructions will be given when dealing with the tissues in detail. (The durations here quoted appear to me exaggerated, except for very voluminous specimens.)

A little acetic or formic acid (0·5 to 1 per cent.) may frequently with advantage be added to the solutions just before using.

If solutions made with pure water be used, they must be kept protected from the light during the immersion of tissues. This precaution is not necessary if Flemming's or Hermann's solution be used. If the immersion is to be a long one the tissues must be placed with the solution in well-closed vessels, as osmium is very volatile.

The principle of combining osmium solutions with alcohol is due to RANVIER ET VIGNAL (RANVIER, *Leç. d'Anat. Gén.*, " App. term. des muscles de la vie org.," p. 76; VIGNAL, *Arch. de Physiol.*, 1884, p. 181). They take equal volumes of 1 per cent. osmic acid and 90 per cent. alcohol (freshly mixed). They wash out in 80 per cent. alcohol, then wash with water and stain for forty-eight hours in picro-carmine or hæmatoxylin. Viallanes has applied this method to the histology of insects.

KOLOSSOW (*Zeit. f. wiss. Mik.*, v, 1, 1888, p. 51) has recommended a 0·5 per cent. solution of osmium in 2 or 3 per cent. solution of nitrate or acetate of uranium, as having a greatly enhanced penetrating power.

He has more lately (op. cit., ix, 1, 1892, p. 39) recommended for the same reason a mixture of 50 c.c. absolute alcohol, 50 c.c. distilled water, 2 c.c. concentrated nitric acid, and 1 to 2 g. osmium. This mixture is said to keep indefinitely in a cool place.

MANN (*Zeit. f. wiss. Mik.*, xi, 4, p. 481) recommends a freshly-prepared mixture of equal volumes of 1 per cent. osmic acid solution and saturated solution of corrosive sublimate in normal salt solution (for nerve centres).

38. After-treatment.—The excess of osmic acid must be well washed out before proceeding to any further steps in preparation; water should be used for washing. Notwithstanding the greatest care in soaking, it frequently happens that some of the acid remains in the tissues, and causes them to over-blacken in time. To obviate this it is necessary to wash them out in ammonia-carmine or picro-carmine, or to soak them for twenty-four hours in a solution of bichromate of potash (Müller's solution or Erlicki's will do), or in 0·5 per cent. solution of chromic acid, or in Merkel's solution, or in a weak solution of ferrocyanide of potassium or cyanide of potassium. The treatment with bichromate solutions has the great advantage of highly facilitating staining with carmine or hæmatoxylin. Max Schultze recommended washing, and mounting permanently in acetate of potash; but I believe the virtues attributed to this method are illusory. Fol has recommended treatment with a weak solution of carbonate of ammonia.

But the best plan of all is to properly *bleach* the preparations (see "**Bleaching**"). This is perhaps most conveniently done (as recommended by FOL, BRASS, and OVERTON) by means of *peroxide of hydrogen,* which regenerates the osmium to osmic acid. OVERTON (*Zeit. f. wiss. Mik.*, vii, 1, 1890) finds that bleaching is completed in a few minutes in a mixture of 1 part commercial peroxide of hydrogen with 10 to 25 parts 70 per cent. alcohol. (The commercial peroxide, slightly acidulated with HCl, will keep well in the dark; but the mixture with alcohol must be made fresh for use.) CARAZZI's peroxide of sodium may be found convenient for this purpose. BINET (*Journ. de l'Anat. et de la Physiol.*, xxx, 1894, p. 449) has successfully used permanganate of potash.

The same stains recommended for objects fixed by vapour will be found useful here, with the addition of ammonia-carmine, which is really very useful for strongly fixed specimens. For sections, of course in both cases safranin and the other nuclear anilin stains may be employed with advantage.

39. Characters of the Fixation with Osmic Acid.—In general osmic acid, especially when used in the form of vapour, preserves the forms of cells more faithfully than any other reagent. But there are some drawbacks over and above those before mentioned (§ 27). The penetrating power of the solutions is very low, so that if any but very small pieces of tissue be taken the outer layers become over-fixed before the action of the reagent has penetrated to the deeper layers. Over-fixed cells have a certain homogeneous, glassy or colloid look, owing to all their constituents having been raised by coagulation to so high an index of refraction that little or no detail is visible in them. They stain very badly, or not at all. Such cells are known as "osmicated cells, *osmirte Zellen.*" There is no remedy for this state of things if once it has occurred. For this reason it is important to avoid using stronger solutions than is necessary. The danger of osmication is lessened by using the osmic acid in conjunction with certain other reagents, such as chromic acid. But it is not thereby entirely removed; FLEMMING's mixture, especially the strong formula, will readily osmicate superficial cells if care be not taken. For ordinary histological work osmication of superficial layers is not of much consequence. But for cytological work care should be taken not to draw conclusions as to the structure of cells from osmicated specimens, and attention should be confined to cells four or five layers deeper down, which will generally be found to present the desired intensity of fixation.

Osmic acid stains all fatty structures black; it must therefore be avoided for tissues in which much fat is present; or if not, the preparations must be subsequently very thoroughly bleached.

40. Chromic Acid.—Chromic anhydride, CrO_3, is found in commerce in the form of red crystals that dissolve readily in water, forming chromic acid, H_2CrO_4. These crystals are very deliquescent, and it is therefore well to keep the acid in stock in the shape of a 1 per cent. solution. Care must be taken not to allow the crystals to be contaminated by organic matter, in the presence of which the anhydride is readily reduced into sesquioxide.

Chromic acid is employed in solution either in water or in alcohol.

The most useful strengths in which it is employed in aqueous solution are from 0·1 to 1·0 per cent. for a period of immersion of a few hours (structure of cells and ova). For nerve-tissues weaker solutions are taken, $\frac{1}{50}$th to $\frac{1}{8}$th per cent. for a few hours. Stronger solutions, such as 5 per cent., should only be allowed to act for a few seconds.

The object should be washed out with water before passing into alcohol or staining fluids. *Long* washing in water is necessary to prepare them for staining, except an anilin stain be used. It is possible to wash out in alcohol, and this may be useful in special cases, but in general I think the practice is not to be recommended. It is well to wash for many hours in *running* water.

Tissues that have been fixed in chromic acid are best stained in aqueous solutions, as water does not appear to have an injurious effect on them; the acid appears to enter into some chemical combination with the elements of the tissues, forming with them a compound that is not affected either physically or chemically by water. The best stain to follow chromic acid is hæmatoxylin, or, for sections, some anilin stain. But the previous washing out with water must be very thorough if good results are to be insured; it may take days.

Chromic acid is not a very penetrating reagent, and for this reason, as well as for others, is seldom used pure, but plays an important part in the mixtures described below, of which the chief is certainly the mixture of Flemming. A chief objection to the use of chromic acid is that *it precipitates certain of the liquid albuminoids of tissues in the form of filaments or networks,* which are often of great regularity, and *simulate structural elements of the tissues.* This objection applies to all mixtures into which chromic acid enters.

41. *Action of light on alcohol containing chromic objects.*—When objects that have been treated by chromic acid or a chromate are put into alcohol for hardening or preservation, it is found that after a short time a fine precipitate is thrown down on the surface of the preparations, thus forming a certain obstacle to the further penetration of the alcohol. Previous washing by water does not prevent the formation of this precipitate, and changing the alcohol does not prevent it from forming again and again. It has been found by Hans Virchow (*Arch. f. mik. Anat.*, Bd. xxiv, 1885, p. 117) that the formation of this precipitate may be entirely prevented by simply keeping the preparations in the dark. The alcohol becomes yellow as usual (and should be changed as often as this takes place), but no precipitate is formed. If this precaution be taken, previous washing with water may be omitted, or at all events greatly abridged.

The brownish-green colour of chromic objects may be removed by treating them with peroxide of hydrogen (Unna, in *Arch. f. mik. Anat.*, Bd. xxx, 1887, p. 47; cf. *Journ. Roy. Mic. Soc.*, 1887, p. 1060; and see the instructions for bleaching osmic acid preparations at the end of § 39).

42. Chromic Acid and Spirit.—A mixture of 2 parts of $\frac{1}{6}$ per cent. chromic acid solution with one part of methylated spirit was much used by Klein in his investigations into the structure of cells and nuclei, and found to give better results than the ordinary reagents (including even osmic acid). Hæmatoxylin was used for staining.

The addition of alcohol to augment the penetrating power of chromic acid seems to be a step in the right direction, and it is matter for surprise that such mixtures are not more used. The alcohol should be added to the acid in aqueous solution, as if strong alcohol be added to crystals of chromic anhydride, a very violent reaction is set up. The mixture should be kept in the dark.

43. Chromo-acetic Acid (Flemming, *Zellsbz. Kern u. Zellth.*, p. 382).

Chromic acid .	0·2 to 0·25 per cent.
Acetic acid . .	0·1 per cent., in water.

Flemming finds this the best reagent for the study of the *achromatic* elements of karyokinesis. (Flemming wrote this in 1882, and I doubt whether it would now hold good.) Stain with hæmatoxylin (the preparations are *not* favorable for staining with safranin or other coal-tar colours).

I can recommend as a good fixing and hardening mixture for Annelids in general, and probably for other forms, the following fluid due to EHLERS (I do not know whether it has been published elsewhere):—To 100 c.c. of chromic acid of 0·5 to 1 per cent. add from 1 to 5 drops of glacial acetic acid. The proportion of acetic acid indicated is sufficient to counteract any tendency to shrinkage due to the chromic acid.

44. Chromo-formic Acid (RABL, *Morph. Jahrb.*, x, 1884, pp. 215, 216).—Four or five drops of concentrated formic acid are added to 200 c.c. of 0·33 per cent. chromic acid solution. The mixture must be freshly prepared at the instant of using. Fix for twelve to twenty-four hours, wash out with water, harden in alcohol, stain with hæmatoxylin or safranin. For the study of karyokinesis. This is acknowledged to be one of the very best reagents for the purpose.

45. Chromo-osmic Acid (MAX FLESCH, *Arch. f. mik. Anat.*, xvi, 1879, p. 300).—This mixture (osmic acid 0·10, chromic acid 0·25, water 100·0) originally introduced for the preparation of the auditory organ of vertebrates, is of general application. It does not require to be kept in the dark. Objects may remain in it for twenty-four or thirty-six hours without risk of the osmic acid over-blackening them. Flemming found it to preserve nuclear figures well, but the preparations are pale, and difficult to stain well. He finds that the action of the mixture is improved (for nuclear figures) by the addition of acetic, formic, or other acid. This addition brings out the figures more sharply, and has the further advantage of allowing of a sharper stain with hæmatoxylin, picro-carmine, or gentian violet. He recommends the following formula, which may be considered to have superseded Max Flesch's.

46. Chromo-aceto-osmic Acid (FLEMMING, *Zellsubstanz, Kern und Zelltheilung*, 1882, p. 381) FIRST OR WEAK formula.—

Chromic acid . .	0·25 per cent.	In water.
Osmic acid . .	0·1 per cent.	
Glacial acetic acid .	0·1 per cent.	

The best results (as regards faithfulness of fixation) are obtained with this mixture when it is allowed to act for only a short time (about half an hour).

But it may, without inconvenience, be allowed to act for many hours or days, or according to some workers, even weeks or months. Wash out, very thoroughly, in water. Stain with hæmatoxylin, if you wish to stain *in toto* (staining in this way with other reagents is possible, but very difficult, and not be recommended). Stain sections with safranin, or other anilin, or with hæmatoxylin or Kernschwarz.

To make up this mixture with the usual stock solutions, you take:

Chromic acid of 1 per cent. . .	25 volumes.
Osmic acid of 1 per cent. . .	10 ,,
Acetic acid of 1 per cent. . .	10 ,,
Water	55 ,,

If you keep your osmium in 2 per cent. solution in chromic acid of 1 per cent., as I have recommended, you will have to take only 20 vols. of chromic acid, 5 of your osmium solution, and 65 of water. See also the remarks on the deterioration of these solutions by keeping, in the next §.

It has been already stated more than once that Flemming's solution is, with the exception of Hermann's solution, probably the very best fixing reagent in general yet discovered. It has, however, been criticised. Faussek (*Zeitschr. f. wiss. Zool.*, Bd. xlv, 1887, pp. 694, *et seq.*) found it totally inapplicable to the histology of the intestine of insects. He states that it caused the intima to disappear, and the cells to run together into a compact mass. Arnold (*Arch. f. mik. Anat.*, Bd. xxx, 1887, p. 205) states that it does not preserve cell-bodies faithfully. And A. Kotlarewsky (*Mitth. d. naturf. Ges. Bern.*, 1887; cf. *Zeit. f. wiss. Mik.*, iv, 3, 1887, p. 387) found that it preserved the forms of nerve-cells (spinal ganglia) less faithfully than any of the reagents tried. I have not, myself, been struck by any defect in the preservation of cytoplasmic structures in my preparations made by this reagent.

It is not necessary in all cases to observe the exact proportions of the ingredients in this mixture. Fol (*Lehrb. d. vergl. mik. Anat.*, 1884, p. 100) recommends the following:

1 per cent. chromic acid	25 vols.
1 per cent. osmic acid	2 ,,
2 per cent. acetic acid	5 ,,
Water	68 ,,

That is to say, a mixture much weaker in osmium than Flemming's. In the *Traité des Méthodes Techniques*, &c., Lee et Henneguy, 1887, I recommended this mixture, as giving better results in general, but am now inclined to think that, at all events as regards fidelity of fixation, it is a step in the wrong direction. Fol's formula has the advantage of allowing better staining with carmine, that is all.

A mixture still weaker than this in osmium, viz. with 1 vol. osmium solution instead of 2, has been recommended by CORI (*Zeit. f. wiss. Mik.*, vi, 1, 1890, p. 441), but I still adhere to the opinion above expressed.

47. Chromo-aceto-osmic Acid (FLEMMING, *Zeit. f. wiss. Mik.*, 1, 1884, p. 349), SECOND OF STRONG formula.—

1 per cent. chromic acid	15 parts.
2 per cent. osmic acid	4 ,,
Glacial acetic acid	1 ,,

If 2 per cent. osmium solution should not be at hand, you may conveniently make the mixture by taking—

10 per cent. chromic acid	15 parts.
1 per cent. osmic acid	80 ,,
Glacial acetic acid	10 ,,
Water	95 ,,

REESE LIBRARY OF THE UNIVERSITY OF CALIFORNIA

If this mixture be kept in stock in large quantities, it may go bad, probably on account of the large proportion of organic acid contained in it. I therefore recommend that it be made up from time to time from stock solutions, in which the osmium is kept separate from the acetic acid. The proportions being as follows:

CrO_3	0·15
Os.	0·08
Acid. acet.	1·00
Aq.	19·00

You may make up and keep separately—

(A)	1 per cent. chromic acid	11 parts.
	Distilled water	4 ,,
	Glacial acetic acid	1 ,,

and (B) a 2 per cent. solution of osmium in 1 per cent. chromic acid solution, and when required, mix four parts of A with one of B; or, of course, if you prefer it, you may keep the osmic and chromic acid ready mixed in the proportions given, and add 5 per cent. of acetic acid at the moment of using.

In any case, it is better not to make up very large quantities of the mixture at once, as osmium being very volatile it will be found that solutions that have been long in use no longer contain the proper proportion of that ingredient, and the hardening action being thus weakened the swelling action of the acetic acid will be insufficiently controlled.

I am convinced that for some purposes there is advantage in diminishing notably the proportion of acetic acid.

Merk (*Densch. d. Math. Naturw. Cl. d. K. Acad. d. Wiss. Wien*, 1887; cf. *Zeit. f. wiss. Mik.*, v, 2, 1888, p. 237) proposes to make up separately (A)

2 per cent. chromic acid	7·5 parts.
Water	3·5 „
Acetic acid	1 „

and (B), some 1 per cent. osmium solution, and to mix for use 12 parts of A with 8 of B. But this plan leaves you in the old difficulty of keeping your osmium in aqueous solution.

It does not appear necessary to observe the exact proportions of the ingredients of these mixtures, a certain latitude is allowable. Thus CARNOY (*La Cellule*, 1, 2, 1885, p. 211) has employed a mixture one third stronger in osmium and twice as strong in chromic acid, viz.

Chromic acid of 2 per cent. (or even stronger)	45 parts.
Osmic acid of 2 per cent	16 „
Glacial acetic acid	3 „

PODWYSSOZKI recommends (for glands especially) the following modification:

1 per cent. Cro.$_3$ dissolved in 0·5 per cent. solution of corrosive sublimate	15 c.c.
2 per cent. osmium solution	4 c.c.
Glacial acetic acid	6 to 8 drops.

The sublimate is said to augment the penetration of the osmium, but is unfavorable to staining. The proportion of acetic acid is reduced in order to avoid swelling of the tissue-elements (ZIEGLER'S *Beiträge z. path. Anat.*, i, 1886; cf. *Zeit. f. wiss. Mik.*, iii, 3, 1886, p. 405).

The strong formula was recommended by FLEMMING in the first instance merely for a very special purpose, the hunting for karyokinetic figures, and not for general purposes. Further experience has shown that it is applicable to general purposes, and is in many cases considerably superior to the weak formula. But it is not suited to all objects, and FLEMMING has lately pointed out that some workers have used it for purposes for which it is not fitted.

Arnold, in the place quoted in the last paragraph, says that it is to be avoided if you wish to demonstrate the structure of certain nuclei (of wandering cells); and the other objections there quoted as applying to the weak formula are intended to apply more or less to the present formula. It will be well not to attach too much importance to them. Let delicate structures be fixed for twenty-four hours or more, washed in running water for an hour, and in successive alcohols for twenty-four hours, sectioned, and stained with safranin or gentian violet, and there will be little complaint of defective preservation.

The strong mixture does not brown tissues more than the weak mixture, but rather less.

Fat is blackened by these mixtures; but the blackened fat can be entirely dissolved out of the tissues by treating them for a few hours with turpentine that has been exposed to sunlight for an hour or two (*see* Flemming in *Zeit. f. wiss. Mik.*, vi, 1, 1889, p. 39; and vi, 2, 1889, p. 178).

The remarks on over-fixation made in § 39 apply to both these mixtures, and more especially to the stronger one. For staining see last §.

48. Platino-aceto-osmic Acid (HERMANN'S **solution**). This *extremely important* reagent is historically a modification of Flemming's solution, platinum chloride being taken instead of chromic acid. See § 65.

49. Nitric Acid (ALTMANN, *Arch. Anat. u. Phys.*, 1881, p. 219).

Altmann employs dilute nitric acid, containing from 3 to 3½ per cent. pure acid. Such a solution has a sp. gr. of about 1·02; an aræometer may conveniently be used to determine the concentration of the solution. Stronger solutions have been used, but do not give such good final results. After extensive trial I am convinced that ALTMANN'S solution is much too weak a reagent for general work, and should be discarded.

His (*ibid.*, 1877, p. 115) recommended a 10 per cent. solution. Flemming at one time employed solutions of 40 to 50 per cent. for the ova of invertebrates. This of course has the advantage of a very rapid fixing action.

Nitric acid has the valuable property of hardening yolk without making it brittle. But, for general purposes at all events, the pure nitric acid solutions may be considered to be superseded by Perenyi's chromo-nitric acid mixture (below, next §).

50. Chromo-nitric Acid (PERENYI'S formula, *Zool. Anzeig.*, v, 1882, p. 459).—

4 parts 10 per cent. nitric acid.
3 parts alcohol.
3 parts 0·5 per cent. chromic acid.

These are mixed, and after a short time give a fine violet-coloured solution.

The objects are immersed for four to five hours, and then passed through 70 per cent. alcohol (twenty-four hours), strong alcohol (some days), absolute alcohol (four to five days). They are then fit for cutting. The advantage of the process is, amongst others, that segmentation spheres and nuclei are perfectly fixed, the ova do not become porous, and cut like cartilage.

Chromo-nitric acid is not only an embryological reagent, and a very important one, but also an admirable one for general work. I have found it altogether excellent for preserving marine organisms, especially large forms. Strong alcohol need only be used if the objects are destined to be sectioned.

For a special formula for embryological purposes, see Part II, "Embryological Methods."

51. Picro-chromic Acid (*Fol. Lehrb.*, p. 100).—

Picric acid, sol. sat. in water	10 vols.
1 per cent. chromic acid solution . . .	25 „
Water	65 „

At the instant of using, you may add 0·005 of osmic acid, which makes the action more energetic. Wash with water (hot, nearly boiling water is best), and then with alcohol. Fol says, "This reagent hardens tissues admirably, without hindering staining in any way; but it is not very penetrating and fixes slowly." I find it gives very good results with Annelids.

I have seen Fol's formula, with the addition of a trace of acetic acid, quoted as "liquid of Haensel"—I know not with what justification.

52.—Chromic Acid and Platinic Chloride (*Merkel's solution;* from *Mitth. Zool. Stat. Neapel,* 1881, p. 11).—Equal volumes of 1·400 solution of chromic acid and 1·400 solution of platinic chloride ($PtCl_4$). Objects should remain in it for several hours or even days, as it does not harden very rapidly. After washing out with alcohol of 50 per cent. to 70 per cent., objects stain excellently, notwithstanding the admixture of chromic acid. This is a very delicate and admirable fixative. If objects that have been fixed by osmium be put into it for some hours, blackening is effectually prevented.

Salts.

53. Bichromate and Cupric Sulphate Mixture (KULTSCHITZKY, *Zeit. f. wiss. Mik.*, iv, 3, 1887, p. 348).—A saturated solution of bichromate of potash and sulphate of copper in 50 per cent. alcohol, to which is added at the instant of using a little acetic acid, five or six drops per 100 c.c.

To make the solution, add the finely powdered salts to the alcohol in excess, and leave them together *in total darkness,* for twenty-four hours.

Fix for twelve to twenty-four hours *in the dark,* otherwise the salts will be precipitated. Then treat with strong alcohol for twelve to twenty-four hours, and make sections.

The rationale of this mixture is stated to be that it fixes tissues faithfully, without causing the production of the delusive reticular precipitates of albuminoids which we have mentioned as being produced by chromic acid—that is the part played by the bichromate and sulphate; and that it also fixes faithfully the chromatin of nuclei—that is the part played by the organic acid.

53a. For LINDSAY-JOHNSON'S **Bichromo-osmic Mixture**, which may be used with excellent results as a gentle fixative, see § 97.

54. The Chromates are useful as hardening rather than fixing agents. They have a very mild and even action on tissues, but are not at all penetrating and act very slowly. They may still be found useful for fixing certain tissues, some of those of Mollusca for example. For mixtures that may be used for such a purpose, *see* the chapter on HARDENING AGENTS.

55. Cupric Sulphate.—Not of general utility. *See* "*Siphonophora.*"

56. Alum.—Alum has been used for fixing purposes. After an extended experience of it, I only quote it in order to recommend that it be avoided at all costs.

CHAPTER V.

FIXING AGENTS. CHLORIDES, ORGANIC ACIDS, AND OTHERS.

Chlorides.

57. Bichloride of Mercury (Corrosive Sublimate).—Corrosive sublimate is stated in the books to be soluble in about sixteen parts of cold and three of boiling water. It will probably be found that the aqueous solution contains about 5 per cent. of the sublimate at the temperature of the laboratory. It is more soluble in alcohol than in water, and still more so in ether. Its solubility in all these menstrua is augmented by the addition of hydrochloric acid, ammonious chloride, or camphor. With sodium chloride it forms a more easily soluble double salt; hence sea water may dissolve as much as 15 per cent., and hence the composition of the liquid of Lang.

The simple aqueous solutions frequently deteriorate in even a short time through the formation of a pulverulent precipitate. The nature of this precipitate is unknown to me, and I have been unable to find any certain means of preventing its formation. Thinking that it may be due in part to ammonia derived from the air, I have lately been in the habit of adding a little nitric acid to my solutions, and certainly have found that they thus keep much better. The addition of one drop of nitric acid for each cubic centimetre of sublimate solution has already, I have since found, been recommended by Frenzel (see Mann, in *Zeit. f. wiss. Mik.*, xi, 4, 1894, p. 480). In any case, for work in which it is desired to obtain as energetic a fixing action as possible, it is well to use only freshly made up solutions.

For fixing, corrosive sublimate may be, and very frequently is, used pure; but in most cases a finer fixation will be obtained if it be acidified with acetic acid, say about 1 per cent. of the acid. I find that a *saturated solution in* 5 *per cent. acetic acid* is a very good formula for *marine animals*. Van Beneden has recommended a saturated solution in 25 per cent. acetic acid.

It is sometimes advisable to take the most concentrated solution obtainable. The cold saturated aqueous solution will suffice in most cases; but for some very contractile forms (coral polypes, Planaria), a concentrated solution in warm or even boiling water should be employed. For Arthropoda the alcoholic solution is frequently indicated. Delicate objects, however, may require treatment with weak solutions. HARTING found solutions of 0·2 to 0·5 per cent. suitable for blood-corpuscles, and PACINI's fluids are much of the same strength. For these see the chapter on Examination Media.

Objects should in all cases be removed from the fixing bath as soon as fixed, that is, in other words, as soon as they are seen to have become opaque throughout, which is practically as soon as they are penetrated by the liquid. Small objects are fixed in a few minutes. I have found that a "salivary" gland of the larva of *Chironomus* is thoroughly fixed in three seconds.

Wash out with water or with alcohol. I consider alcohol always almost preferable. Alcohol of about 70 per cent. may be taken. The extraction of the sublimate is hastened by the addition of a little camphor to the alcohol. Or, better, a little tincture of iodine may be added to the liquid, either alcohol or water, used for washing, and the liquid changed until it no longer becomes discoloured by the objects. It is important that the sublimate be thoroughly removed from the tissues, otherwise they become brittle. They will also become brittle if they are kept long in alcohol.

It has been advised that solution of iodine in potassium iodide be taken instead of tincture of iodine for the washing out. But that is quite wrong, iodine in potassium iodide precipitates corrosive sublimate. I am much obliged to my friend Prof. GILSON for calling my attention to this point.

It may happen that if the extraction of the excess of sublimate from the tissues in bulk has been insufficient, crystals of sublimate may form in the sections after they have been mounted in balsam. This may easily be prevented by treating the sections themselves with tincture of iodine for a quarter of an hour before mounting. This will do away with the necessity of treating the tissues in bulk with iodine, which is frequently a very long process (unless it is desired to keep the material for a long time in alcohol before making the sections).

You may stain in any way you like. Carmine stains are peculiarly brilliant after sublimate (owing, it has been said, to the formation of mercuric carminate). It is not necessary that the objects be thoroughly washed out before staining; the staining processes themselves may be made to constitute a part of the washing-out process.

It must be remembered that the solutions must not be touched with iron or steel, as these produce precipitates that may hurt the preparations. To manipulate the objects, wood, glass, or platinum may be used; for dissecting them, hedgehog spines, or quill pens.

When properly employed, sublimate is *for general work* undoubtedly a fixing agent of the very highest order. It is applicable to most classes of objects. It is perhaps less applicable, in the pure form, to Arthropods, as it possesses no great power of penetrating chitin. For *cytological work* it is, according to my experience, not to be trusted, and not to be recommended.

58. Corrosive Sublimate (Lang's formula, '*Zool. Anzeiger,*' 1878, i, p. 14). For *Planaria.*—Take—

Distilled water . . .	100 parts by weight.
Chloride of sodium . .	6 to 10 parts.
Acetic acid	6 to 8 ,,
Bichloride of mercury . .	3 to 12 ,,
(Alum, in some cases . .	½).

Second formula (*ibid.*, 1879, ii, p. 46).—Make a concentrated solution of corrosive sublimate in picro-sulphuric acid, to which has been added 5 per cent. of acetic acid.

59. Other Simple Solutions.—A solution containing 5 g. sublimate, 0·5 g. sodium chloride, and 100 c.c. water, has been quoted as "solution of Gaule."

Keiser's solution consists of 10 g. sublimate, 3 g. glacial acetic acid, and 300 g. distilled water (from *Zeit. f. wiss. Mik.*, xi, 3, p. 378).

M. Heidenhain has recommended a 0·5 per cent. solution of sodium chloride saturated while hot with sublimate.

60. Gilson's **Mercuro-nitric Mixture.**—I am indebted to Prof. Gilson for kindly sending the latest formula (1895), which is as follows:

Nitric Acid of 46° strength (this would be sp. gr. 1·456, or 80 per cent., nearly)	78 c.c.
Glacial acetic acid	22 ,,
Corrosive sublimate	95 to 100 grms.
60 per cent. alcohol	500 c.c.
Distilled water	4400 ,,

When required *for marine animals* add a few crystals of iodine, which will prevent the formation of precipitates of sea salts. If in any case the preparations should show a granular precipitate, due probably to an abundance of phosphates in the tissues, the precipitate may be removed by washing with water containing a little tincture of iodine (*not* iodide of potassium, which would precipitate the sublimate).

I have tried this mixture and find that it affords in general a faithful and delicate fixation, and gives to tissues an excellent consistency. Objects may remain in it for a considerable time without hurt. Tissues are left in a state very favorable for staining. The liquid has a high degree of penetration. A treatment for a few days with it will serve to remove the albumen from the ova of Batrachians. This liquid may be recommended to beginners, as it is very easy to work with. For some objects, as I have found, the proportion of sublimate may be increased with advantage.

61. Rabl's **Picro-sublimate** (*Zeit. f. wiss. Mik.*, xi, 2, 1894, p. 165).—Sublimate, saturated solution in water, 1 vol.; a similar solution of picric acid, 1 vol.; distilled water, 2 vols. Embryos may be left in it for twelve hours, washed for two hours in water, and brought into weak alcohol.

62. Mann's **Picro-sublimate** (*op. cit.*, xi, 4, 1895, p. 480).—1 per cent. of picric acid with or without 1 per cent. of tannin in a saturated solution of sublimate in normal salt solution.

The same author's **Alcoholic Picro-sublimate** (*Anat. Anz.*, 8, 1893, pp. 441—443) consists of absolute alcohol 100 c.c., picric acid 4 grms., sublimate 15 grms., tannin 6 to 8 grms. The tannin is added in order to prevent excessive hardening.

The same author's **Mercurio-osmic Mixture** has been given (§ 37).

Vom Rath's **Picro-sublimate** (*Anat. Anz.*, xi, 9, 1895, p. 286).

—Cold saturated solution of picric acid, 1 part; hot saturated solution of sublimate, 1 part; glacial acetic acid, ½ to 1 per cent. Fix for several hours and bring direct into alcohol.

The same author's **Picro-sublimate-osmic Mixture** (loc. cit.) consists of the above with the addition of 10 per cent. of 2 per cent. osmic acid solution.

63. ZENKER's **Mixture** (*Münchener med. Wochenschr.*, 24, 1894, p. 534; quoted from MERCIER, *Zeit. f. wiss. Mik.*, xi, 4, 1894, p. 471, where will be found minute instructions for using it). Five per cent. of sublimate and 5 per cent. of glacial acetic acid, dissolved in solution of MÜLLER. Fix for several hours, wash out with water, treat the tissues in bulk, or the sections, with alcohol containing tincture of iodine.

63a. FOÀ's **Mixture** (*Quart. Journ. Mic. Sci.*, 1895, p. 287; *Journ. Roy. Mic. Soc.*, 1895, p. 486). Equal parts of saturated solution of sublimate in normal salt solution, and of liquid of Müller, or 5 per cent. solution of bichromate.

The rationality of these mixtures is not apparent. The addition of the chrome salts is unfavorable to staining with carmine or hæmatin.

64. Chloride of Platinum (Platinic Chloride, $PtCl_4$**).**—An *extremely valuable* reagent, originally introduced for the study of karyokinesis, but of general application. RABL, to whom we owe the introduction of this agent, employed an aqueous solution of 1·300. The objects remain in it for twenty-four hours, and are then washed with water, hardened in alcohol, and sectioned. Rabl stained with Delafield's hæmatoxylin, or with safranin.

The action of platinum chloride is similar to that of gold chloride, with the advantage that there is no blackening of the preparations. Rabl finds it give better results (for the study of karyokinesis) than any other reagent except chromoformic acid (§ 44). It causes a slight shrinkage of the chromatin elements, a condition that renders the granules of Pfitzner and the longitudinal division of the elements very distinctly visible (see Rabl's well-known paper in *Morph. Jahrb.*, Bd. x, 1884, p. 216).

Platinum chloride is an extremely deliquescent salt, and for this reason had better be procured in solution. Ten per cent. solutions are found in commerce.

For MERKEL's solution (chromo-platinic mixture) *see ante*, § 52.

For RABL's **Platino-sublimate Mixture**, see *Embryological Methods.*

65. Platino-aceto-osmic Mixture (HERMANN, *Arch. f. mik. Anat.*, xxxiv, 1889, p. 58).—The author obtained excellent results by substituting 1 per cent. platinic chloride for the chromic acid in Flemming's *strong* formula for chromo-aceto-osmic acid (§ 47), the other ingredients either remaining as before, or the osmium being diminished one half. Thus, 1 per cent. platinic chloride 15 parts, glacial acetic acid 1 part, and 2 per cent. osmic acid either 4 parts or only 2 parts. Hermann found that protoplasmic structures are thus better preserved than with the chromic mixture. It was noted above (§ 40) that a chief objection to the use of chromic acid is that it precipitates certain of the liquid albuminoids of tissues in the form of filaments or networks, which are often of great regularity, and simulate structural elements of the tissues. *This platinum chloride does not do.*

The after-treatment and staining should be the same as for objects treated with Flemming's solution.

The remarks in § 47, as to the deterioration of Flemming's solution by evaporation of osmium, apply with equal force to Hermann's mixture.

After considerable experience of this reagent I find that it has the advantage of giving more colourless preparations, and, it may be in some cases, a more delicate fixation. But the fixation is certainly not in all cases superior, the hardening is less energetic, and in some cases not so energetic as may be desired. As this is the most expensive of all reagents, I am glad to find that *for general work* there is no valid reason for supposing that it ought to take the place of Flemming's mixture.

66. Palladium Chloride.—Palladium chloride has been recommended by experienced workers It is used in solutions of 1:300, 1·600, or 1·800 strength, for from one to two minutes. CATTANEO recommends it as being the best of fixatives for Infusoria. Tissues are impregnated and coloured brown by it. For small objects one or two minutes will suffice for fixation.

This salt is found in commerce in the solid state. To dissolve it, take 10 grammes of the salt, one litre of water, and four to six drops of hydrochloric acid. Solution will be effected in twenty-four hours.

FRENKEL (*Anat. Anz.*, viii, 1893, p. 538; *Zeit. f. wiss. Mik.*, x, 2, 1893, p. 243) recommends for connective tissue a mixture of 15 parts 1 per cent.

palladium chloride, 5 parts 2 per cent. osmic acid, and a few drops of acetic acid.

67. Perchloride of Iron (FOL, *Zeit. f. wiss. Zool.*, Bd. xxxviii, 1883, p. 491; and *Lehrb. d. vergl. mik. Anat.*, p. 102).—Fol recommends 1 vol. of *Tinct. Ferri Perchlor.* P. B. diluted with 5 to 10 vols. of 70 per cent. alcohol. This reagent has too many defects to be recommendable except in very special cases.

The tincture diluted with 3 to 4 vols. of either alcohol or water has been recommended for fixing medullated nerve by PLATNER (*Zeit. f. wiss. Mik.*, vi, 2, 1889, p. 187).

Organic Acids, and other Agents.

68. Acetic Acid.—The place of honour amongst organic acids considered as fixing agents appears rightfully to belong to this old-fashioned reagent. In the first edition of this work it was merely stated that acetic and formic acid "are useful and well-known fixatives of nuclei. Flemming, who has made a special investigation of their action, finds (*Zell-substanz*, &c , p. 380) that the best strength is from 0·2 to 1 per cent. Strengths of 5 per cent. and more bring out the nuclein structures clearly at first, but after a time cause them to swell and become pale, which is not the case with the weaker strengths" (*ibid.*, p. 103). It must now be stated that, thanks to v. BENEDEN, the *strong* acid has become established as a most precious fixative of the most varied zoological objects. It is particularly applicable to very contractile objects, such as many Vermes, Cœlenterata, and Nudibranchs; it kills with the utmost rapidity, *and has a tendency to leave them fixed in the state of extension.* The *modus operandi* is in general as follows:—Pour glacial acetic acid in liberal quantity over the organisms, leave them until they are penetrated by it—which should be in five or six minutes, as the strong acid is a highly penetrating reagent—and wash out in frequent changes of alcohol of gradually increasing strength. Some persons begin with 30 per cent. alcohol, but this appears to me rather weak, and I think 70 per cent. or at least 50 per cent alcohol should be preferred.

In the *Traité des Méth. Techn.*, 1887, I stated that the reason why glacial acetic acid was not more used was that it did not faithfully preserve delicate histological and cytological detail. I now believe that if the instructions above given be followed, in particular as regards the employment of the *glacial* acid, and the washing out with somewhat strong alcohol, even delicate detail will generally be found well preserved. I see no reason why other energetic reagents should not be combined with the glacial acetic acid if desired. Dr. Lindsay Johnson (*in. litt.*) has found that one of the best fixatives for retina is a mixture of equal parts glacial acetic acid and 2 per cent. osmic acid. S. Lo Bianco adds to the concentrated acid one-tenth of a 1 per cent. solution of chromic acid. He finds that even this small proportion of chromic acid serves to counteract in a marked degree the softening action of the acetic acid.

69. Acetic Alcohol (Carnoy, *La Cellule*, t. iii, 1, 1886, p. 6; and *ibid.*, 1887, 2, p. 276; v. Beneden et Neyt, *Bull. Ac. roy. d. sci. de Belg.*, t. xiv, 1887, p. 218; Zacharias, *Anat. Anz.*, iii, Jahrg., 1, 1888, pp. 24—27; v. Gehuchten, *ibid.*, 8, p. 227).—Carnoy has given two formulæ for this important reagent. The first is—

Glacial acetic acid . . .	1 part.
Absolute alcohol	3 parts.

The second is—

Glacial acetic acid . .	1 part.
Absolute alcohol	6 parts.
Chloroform	3 ,,

The addition of chloroform is said to render the action of the mixture more rapid.

V. Beneden and Neyt take equal volumes of glacial acid and absolute alcohol.

Zacharias takes—

Glacial acetic acid . . .	1 part.
Absolute alcohol	4 parts.
Osmic acid	A few drops.

Acetic alcohol is one of the most penetrating and quickly-acting fixatives known. It preserves nuclei admirably, and admits of admirable staining in any way that may be preferred. It was imagined by all of the authors quoted for the study of karyokinesis in the ova of *Ascaris*,—proverbially one of the most difficult objects to fix,—but it is applicable to tissues in general. You may wash them out with alcohol and treat them afterwards in any way that may be preferred. It will be well, however, to avoid treatment with water as much as possible.

70. Formic Acid may be used dilute in the same way as acetic acid (*supra*, § 68). It is probable that it might also take the place of acetic acid in the concentrated form, but I am not aware of any experiments in this direction.

71. Chloride and Acetate of Copper (*Ripart et Petit's Liquid*, Carnoy, *La Biologie Cellulaire*, p. 94).

Camphor water (not saturated)	75 grammes.
Distilled water	75 ,,
Crystallised acetic acid	1 gramme.
Acetate of copper	0·30 ,,
Chloride of copper	0·30 ,,

This is a very moderate and delicate fixative. I consider that it has not sufficient hardening power for objects that are intended to be dehydrated and mounted in balsam, but is frequently excellent and sometimes indispensable for objects that are to be studied in as fresh a state as possible in aqueous media. Objects fixed in it stain instantaneously and perfectly with methyl green. Osmic acid may be added to the liquid to increase the fixing action. For *cytological researches* this is a most invaluable medium.

72. Acetate of Uranium (Schenk, *Mitth. a. d. Embryol. Inst. Wien*, 1882, p. 95; cf. Gilson, *La Cellule*, 1, 1885, p. 141).—This reagent is very similar in its properties to picric acid. It has a mild fixing action, and a high degree of penetration, which may make it useful for Arthropoda. It may be combined with methyl green, which it does not precipitate.

73. Iodine.—Iodine possesses considerable hardening properties, and a very high degree of penetration. Kent (*Manual of the Infusoria*, 1881, p. 114; *Journ. Roy. Mic. Soc.* (N.S.), iii, 1883, p. 730) has found it to act in a manner almost identical with osmic acid, and in some instances even more efficiently (for fixing Infusoria). His instructions are as follows:—"Prepare a saturated solution of potassic iodide in distilled water, saturate this solution with iodine, filter, and dilute to a brown-sherry colour. A very small portion only of the fluid is to be added to that containing the Infusoria."

Or you may use the solution of Lugol, of which the formula is as follows:

Water	100 parts.
Iodide of potassium	6 ,,
Iodine	4 ,,

Iodine certainly kills cells very rapidly, without deforming them. Personally I have found it very useful for the examination of spermatozoa. Unfortunately I am not acquainted with any nuclear stain that will work well with it.

Very small objects may be instantaneously fixed by means of **vapour of iodine.** Crystals of iodine may be heated in a test-tube till the vapours are given off; then on inclining the tube the heavy vapours may be made to flow over the objects arranged on a slide. The slide should then be warmed to about 40° C. for 2 or 3 minutes in order to evaporate the iodine from the objects, which may then be mounted or otherwise treated as desired (OVERTON, *Zeit. f. wiss. Mik.*, vii, 1, 1890, p. 14).

REESE

74. Picric Acid.—Picric acid should always be employed in the form of a *strong* solution. (That is to say, strong solutions must always be employed when it is desired to make sections or other preparations of tissues with the elements *in situ,* as weak solutions macerate; but for dissociation preparations or the fixation of isolated cells, weak solutions may be taken. Flemming finds that the fixation of nuclear figures is equally good with strong or weak solutions.) The saturated solution is the one most employed. (One part of picric acid dissolves in about 75 parts of cold water; in hot water it is very much more soluble.) Objects should remain in it for from a few seconds to twenty-four hours, according to their size. For Infusoria one to at most two minutes will suffice, whilst objects of a thickness of several millimètres require from three to six hours' immersion.

Picric acid should *always be washed out with alcohol,* as water is hurtful to tissues that have been prepared in it. For the same reason during all remaining stages of treatment, water should be avoided; staining should be performed by means of alcoholic solutions, the only exceptions to this rule being in favour of picro-carmine, which, probably on account of the picric acid contained in it, does not appear to exert so injurious an influence as other aqueous stains, and of methyl green, and some few other aqueous stains that are themselves weak hardening agents. It is one of the advantages of picric acid that, by sufficiently prolonged soaking, it can with certainty be entirely removed from any tissue by means of alcohol.

It has been found by JELINEK (*Zeit. f. wiss. Mik.*, xi, 2, 1894, p. 242) that the extraction of picric acid is greatly quickened by the addition of a base to the wash-alcohol. He recommends carbonate of lithine. A few drops of a saturated solution of the salt in water are added to the alcohol; a slight precipitate is formed. The objects are put into the turbid alcohol, which becomes clear and yellow in proportion as the picrin is extracted. Further quantities of carbonate are added from time to time until the colour has been entirely extracted from the tissues.

Tissues fixed in picric acid can, after removal of the acid by soaking, be perfectly stained in any stain. Mayer's paracarmine, Grenacher's alcoholic borax-carmine, or Mayer's hæmacalcium may be recommended for entire objects.

The most important property of picric acid is its great penetration. This renders it peculiarly suitable for the preparation of chitinous structures. For such objects alcohol of 70 per cent. to 90 per cent. should be taken for washing out, and staining should be done by means of Mayer's cochineal or hæmacalcium.

In very many if not most cases it is advantageous to employ picric acid in the manner suggested by Kleinenberg (*see* below), that is, in combination with sulphuric acid; or with nitric acid, or hydrochloric acid, as suggested by P. Mayer (*see* below).

75. Picro-sulphuric Acid (KLEINENBERG, *Quart. Journ. Mic. Sci.*, April, 1879, p. 208; MAYER, *Journ. Roy. Mic. Soc.* (N.S.), ii (1882), p. 867).—By picro-sulphuric acid, without any qualifying term, I understand a fluid made (following Mayer l. c.) as follows:—Distilled water, 100 vols.; sulphuric acid 2 vols.; picric acid, as much as will dissolve (this will be about 0·25 per cent.; as the picric acid is much less soluble in sulphuric acid solution than in water). This may also, in any case in which confusion is likely to arise, be called "concentrated" or "undiluted picro-sulphuric acid."

By "liquid of Kleinenberg" I understand a mixture suggested by Kleinenberg (*l. c.*), and best made by diluting the concentrated picro-sulphuric acid prepared as above with three times its volume of water.

(Kleinenberg also directed the addition of as much creasote as would mix. This was done with the idea of eliminating the swellings produced in some objects by the liquid, but it has been found not to have the effect attributed to it, and has been abandoned. FOL (*Lehrb.*, p. 100) states that the same end may be attained by adding one third vol. of 1 per cent. chromic acid.)

Of these two formulæ the one commonly employed is that given by Kleinenberg,—the dilute mixture; undiluted picro-sulphuric acid being reserved for objects requiring special treatment, chiefly Arthropods. I may as well say at once that in my opinion this practice is erroneous, for I hold that Kleinenberg's solution is much weaker than is desirable in the majority of cases, and should be reserved for special cases, such perhaps as that for which it was originally proposed, the embryology of the earthworm; and the concentrated solution should be the one taken for general work. *This particularly applies to marine organisms.*

The treatment is the same in either case. "The object to be preserved should remain in the liquid for three, four, or more hours; then it should be transferred, in order to harden it and remove the acid, into 70 per cent. alcohol, where it is to remain five or six hours. From this it is to be removed into 90 per cent. alcohol, where it is to be changed until the yellow tint has either disappeared or greatly diminished."

Warm alcohol extracts the acid much more quickly than cold, without which *weeks* may be required to fully remove the acid from chitinous structures. I call attention here to what was said as to washing out under the head of *picric acid,* viz. that washing out must *never be done with water.* This is a most important point, and one that is not sufficiently attended to. You may stain as directed above for picric acid. You may, of course, stain sections with alcoholic solutions of safranin or the like.

The advantages of picro-sulphuric acid as a fixing agent are, that it kills tissues very rapidly, that it has great penetrating power, that it can be totally soaked out of the structures with alcohol (it is much more easily removed from the tissues than pure picric acid), leaving them in a good condition for staining, and, in the case of marine organisms, that it effectually removes the different salts of sea-water that are present in them.

It has many disadvantages. For vertebrata it should be used with caution, on account of the swelling caused by sulphuric acid in connective tissue. For structures that contain much lime it is not to be recommended, for it dissolves the lime and throws it down as crystals of gypsum in the tissues. (For such structures the picro-nitric or picro-hydrochloric acid is to be preferred.) In numberless cases it produces swellings and maceration. For the preservation of delicate, watery organisms, such as Medusæ, it is an abomination. For cytological researches it should be avoided, as its action on both cytoplasm and nuclei is frequently most injurious. On the whole, I find that for such objects as Arthropoda it is valuable on account of its great penetrating power, the possibility of removing the acid entirely by washing, and the facility thereby given for staining *in toto*. But *for general work, I consider that it is one of the most overrated reagents that ever came into favour through the prestige of authority.*

76. Picro-nitric Acid (Mayer, *Mitth. Zool. Stat. Neapel*, 1881, p. 5; *Journ. Roy. Mic. Soc.* (N.S.), ii, 1882, p. 868).—

Water	100 vols.
Nitric acid (of 25 per cent. N_2O_5)	5 ,,
Picric acid, as much as will dissolve.	

The fluid is used undiluted.

The properties of this fluid are very similar to those of picro-sulphuric acid, with the advantage of avoiding the formation of gypsum crystals, and the disadvantage that it is much more difficult to soak out of the tissues. "Mayer recommends it strongly, and states that with eggs containing a large amount of yolk material, like those of Palinurus, it gives better results than nitric, picric, or picro-sulphuric acid."

77. Picro-hydrochloric Acid (Mayer, *ibid.*).—

Water	100 vols.
Hydrochloric acid (of 25 per cent. HCl)	8 ,,
Picric acid, as much as will dissolve.	

The fluid is used undiluted.

The properties of this fluid are similar to those of picro-nitric acid.

78. Picro-chromic Acid. *See ante*, § 51.

79. Picro-osmic Acid.—Flemming (*Zells. Kern u. Zellth.*, p. 381) has experimented with mixtures made by substituting picric for chromic acid in the chromo-osmic mixtures (*ante*, §§ 46 and 47). The results are identical so far as regards the fixation (of nuclei); but staining is rendered more difficult.

O. vom Rath (*Anat. Anz.*, xi, 1895, p. 289) adds to 200 c.c. of saturated aqueous solution of picric acid, 12 c.c. of 2 per cent. solution of osmic acid, and 2 c.c. of glacial acetic acid.

80. Picro-platinic and Picro-platin-osmic Mixtures.—O. vom Rath (l. c., pp. 282, 285) makes a picro-platinic mixture with 200 c.c. saturated aqueous solution of picric acid, 1 g. of platinic chloride (dissolved in 10 c.c. of water), and 2 c.c. of glacial acetic acid.

The picro-platin-osmic mixture is made by adding to the foregoing 25 c.c. of 2 per cent. osmic acid.

81. Picric Alcohol (Gage, *Proc. Amer. Soc. Micr.*, 1890, p. 120; *Journ. Roy. Mic. Soc.*, 1891, p. 418).—Alcohol (95 per cent.), 250 parts; water, 250 parts; picric acid, 1 part. Fix for about 24 hours, wash out for a day in alcohol of 67 to 70 per cent., and then for a day or longer in alcohol of 75 to 82 per cent.

Other Fixing Agents.

82. Alcohol.—For fixing, only two grades of alcohol are found generally useful—very weak alcohol on the one hand, and absolute alcohol on the other hand. Absolute alcohol ranks as a fixing agent because it kills and hardens with such rapidity that structures have not time to get deformed in the process by the energetic dehydration that unavoidably takes place. Dilute alcohol ranks as a fixing agent in virtue of being of such a strength as to possess a sufficiently energetic coagulating action and yet contain enough water to have but a feeble and innocuous dehydrating action. The intermediate grades do not realise these conditions, and therefore should not be employed alone for fixing. But they may be very useful in combination with other fixing agents (such as corrosive sublimate, chromic acid or nitric acid) by greatly enhancing their penetrating power; 70 per cent. is a good grade for this purpose.

83. One-third Alcohol.—The one grade of weak alcohol that is found generally useful for fixing is one third alcohol, or RANVIER'S ALCOHOL, known in France as "Alcool au tiers," which is the name given to it by Ranvier himself; in Germany as "Drittelalcohol" or "Ranviersche alcohol dilutus"; in Italy as "alcool al terzo." It consists *of two parts of water and one part of alcohol of* 90 *per cent.* (and *not* of absolute alcohol, as was stated by an oversight in the first edition—an error which I have seen copied in more than one place). See the *Traité Technique* of Ranvier, p. 241, *et passim.*

Care should be taken that the alcohol is of the strength specified, as the effects of this reagent depend to a remarkable degree on its strength.

Objects may be left for twenty-four hours in this alcohol; not more, unless there be no reason for avoiding maceration, which will generally occur after that time. You may conveniently stain with picro-carmine, alum-carmine, or methyl green.

This reagent is a very mild fixative. Its hardening action is so slight that it is seldom indicated for the fixing of objects that are intended to be sectioned. Its chief use is for extemporaneous and disassociation preparations.

84. Absolute Alcohol.—This is also a very valuable reagent. It preserves very well the structure of nuclei, which is by no means the case with one-third alcohol. It has over the latter also the advantage of superior penetrating power, being indeed one of the most penetrating of known fixing agents. Mayer finds that boiling absolute alcohol is often the only means of killing certain Arthropoda rapidly enough to avoid maceration brought about by the slowness of penetration of common cold alcohol (especially in the case of Tracheata).

It is important to employ for fixing a very large proportion of alcohol. Alum-carmine is a good stain for small specimens so fixed. For preservation, the object should be put into a weaker alcohol, 90 per cent. or less.

Absolute alcohol is found in commerce. It is a product that it is almost impossible to preserve in use, on account of the rapidity with which it hydrates on exposure to air. Fol recommends that a little quicklime be kept in it. This absorbs part at least of the moisture drawn by the alcohol from the air, and has the further advantage of neutralising the acid that is frequently present in commercial alcohol.

Another plan that I have seen recommended is to suspend strips of gelatin in it. It is stated that by this means ordinary alcohol may be rendered absolute.

Ranvier adopts the following plan for preparing an alcohol absolute enough for all practical purposes. Strong (95 per cent.) alcohol is treated with calcined cupric sulphate, with which it is shaken up and allowed to remain for a day or two. It is then decanted and treated with fresh cupric sulphate, and the operation is repeated until the fresh cupric sulphate no longer becomes conspicuously blue on contact with the alcohol; or until, on a drop of the alcohol being mixed with a drop of turpentine, no particles of water can be seen in it under the microscope. The cupric sulphate is prepared by calcining common blue vitriol in a porcelain capsule over a spirit lamp or gas burner until it becomes white, and then reducing it to powder (see *Proc. Acad. Nat. Sci. Philad.*, 1884, p. 27; *Science Record*, ii, 1884, p. 65; *Journ. Roy. Mic. Soc.* (N.S.), iv, 1884, pp. 322 and 984).

85. Acidulated Alcohol (PAUL MAYER, *Mitth. Zool. Stat. Neapel*, ii, 1881, p. 7).—To 97 vols. of 90 per cent. alcohol, in which is dissolved a small quantity of picric acid, add 3 vols. pure hydrochloric acid. Leave the specimens in the mixture only just long enough to ensure that they are thoroughly penetrated by it. Wash out with 90 per cent. alcohol, the disappearance of the yellow stain of the picric acid being a sign that all the acid is removed.

The use of this mixture is for the *preparation of coarse objects* it is intended to preserve in alcohol. The object of the acid is to prevent both that glueing together of organs by the perivisceral liquid, which is often brought about by the coagulating action of pure alcohol, and the precipitation on the surface of organs of the salts contained in sea-water, which is a hindrance not only to the penetration of the alcohol, but also to subsequent staining.

Whitman (*Journ. Roy. Mic. Soc.* (N.S.), ii, 1882, p. 870) states that "acid alcohol as above prepared loses its original qualities after standing some time, as ether compounds are gradually formed at the expense of the acid." He also states that 70 per cent. alcohol may be taken instead of 90 per cent. for washing out.

86. Formaldehyde (Formol, Formalin, Formalose).—Formaldehyde is the chemical name of the gaseous compound HCOH, obtained by the oxidation of methyl-alcohol. "Formalin" is the commercial name given by SCHERING & Co. to a 40 per cent. solution of this substance in water. "Formol" is the commercial name given to the same solution by MEISTER, LUCIUS, & BRÜNING. And "Formalose" is the name for the same solution adopted by an American firm. (These solutions may now be obtained from dealers in photographic chemicals.) As I have before pointed out (*Anat. Anz.*, xi, 8, 1895, p. 255), the already extensive literature which treats of the anatomical uses of formaldehyde is much confused by inaccurate use of these terms; many writers use them indiscriminately. It is frequently impossible to discover from the statements of an author whether he means such or such a percentage of formaldehyde, or such or such a percentage of the commercial 40 per cent. solution employed by him, the one being of course two and a half times stronger than the other. I think it must be admitted that the proper way of stating the strengths of these solutions is either to state them in terms of formaldehyde, and say so, or to say "formol, or formalin, diluted with so many volumes of water." The present confusion is most inconvenient.

Solutions of formaldehyde sometimes decompose partially or entirely with formation of a white deposit of paraform. FISH says that to avoid this the solutions should be kept in darkened bottles in the cool. The vapour of formaldehyde has a very irritating action on the conjunctiva and mucous membranes, but the effect is transitory, not so injurious as that of osmic acid. It is well not to soil the fingers with the solutions, as formaldehyde hardens the living skin very rapidly.

It was discovered independently by F. BLUM (*Zeit. f. wiss. Mik.*, x, 3, 1893, p. 314) and by HERMANN (*Anat. Anz.*, ix, 4, 1893, p. 112) that formaldehyde possesses most remarkable hardening and preservative properties.

Blum employed formol diluted with ten volumes of water (containing rather less than 4 per cent. of formaldehyde). He found this solution to penetrate rapidly, and to harden voluminous organs such as liver, kidney, brain, more rapidly than alcohol, and that sections were well preserved and susceptible of good staining.

Hermann used a solution containing 0·5 to 1 per cent. of "formalin" (the context shows that 1 per cent. of *formaldehyde* is what is meant, the solution being made by diluting Schering's formalin with forty volumes of water). He found it harden very rapidly, with the remarkable result that the hardened organs preserve *approximately the transparency of life*, and that *pigments are not discoloured*. Since that time these observations have been amply confirmed, and there is no longer any doubt that for the preparation and preservation of *museum specimens* formaldehyde is the most valuable reagent that has been discovered since the introduction of alcohol, to which it is for some purposes infinitely superior (for the employment of formaldehyde in museum work, see Blum, *Zool. Anz.*, xviii, 3, 450).

On account of the confusion in terminology above referred to, it is not at present possible to give precise instructions as to the strengths that have been employed by the different authors for *histological work.* All that can be said is that they will almost certainly be found to lie between the limits of those indicated by Blum and Hermann, that is to say between 0·5 per cent. and 4 per cent. *if the formaldehyde be used pure.* Only one writer (Hoyer, jun., *Anat. Anz.*, ix, 1894, Ergänzungsheft, p. 236; *Zeit. f. wiss. Mik.*, xii, 1, 1895, p. 28) appears to have used concentrated solutions. He states that with such solutions tissues are better preserved than with weak ones, even better preserved than with corrosive sublimate. There is certainly some mistake here. I find that preparations fixed in 13·3 per cent. formaldehyde (formol with two volumes of water) have the cells enormously over-fixed and presenting the homogeneous aspect of osmicated cells.

Experimenting further with weak solutions containing from 2 per cent. to 4 per. cent of formaldehyde, I have found that like the stronger solution mentioned above, these too give a homogeneous, colloid appearance to protoplasm, and have at the same time a marked swelling and vacuolating action. With the 2 per cent. solution the vacuolation is enormous. I have concluded that *used pure* formaldehyde is not at all fitted for cytological work, and should not be employed for that purpose, and I certainly should not think of using it myself, even for general morphological work.

But preparations fixed in a *mixture* of 1 part of formol with two of 1 per cent. chromic acid, and with 4 per cent. of acetic acid added, or in a mixture of one part of formol with four of 1 per cent. platinic chloride, and 2 per cent. of acetic acid added, give an excellent fixation, in some points superior to that of mixture of Flemming or of Hermann.

I certainly think that mixtures of this sort will be found in some cases to give better results than osmio-chromic mixtures, on account of the superior penetration of the formaldehyde on the one hand, and on the other from the fact that formaldehyde, whilst it hardens very rapidly does not over-harden.

Mixtures with alcohol have also been recommended. Lavdowsky (*Anat. Hefte*, iv, 3, 1894, p. 355) gives the following two formulæ:

(1)	Distilled water	20	parts.
	95 per cent. alcohol	10	,,
	Formol	3	,,
	Glacial acetic acid	0·5	,,
(2)	Distilled water	30	parts.
	95 per cent. alcohol	15	,,
	Formol	5	,,
	Glacial acetic acid	1	,,

For the special hardening of the *central nervous system* with formaldehyde see further in Part II.

CHAPTER VI.

HARDENING AGENTS.

87. Introductory.—Generalities concerning the necessity and the practice of hardening have been given in Chap. III, § § 31 and 32. The present chapter will describe some reagents that are specially suited for slow hardening, and much less suited for that rapid hardening distinguished as fixing. It will at the same time describe the manner of employment for prolonged hardening of some of the reagents already described under the head of Fixing Agents. The very special question of the hardening of the central nervous system is merely touched on here, and receives further development in the chapters devoted to *Neurological Methods* in Part II.

Mineral Acids.

88. Chromic Acid.—Chromic acid is generally employed in strengths of $\frac{1}{5}$th per cent. to $\frac{1}{2}$ per cent., the immersion lasting a few days or a few weeks, according to the size and nature of the object. Mucous membrane, for instance, will harden satisfactorily in a few days; brain will require some six weeks.

Large quantities of the solution must be taken (at least 200 grammes for a piece of tissue of 1 centimètre cube, Ranvier). In order to obtain the best results you should not employ portions of tissue of more than an inch cube. For a human spinal cord you should take two litres of solution, and change it for fresh after a few days. Six weeks or two months are necessary to complete the hardening.

The solution should be taken weak at first, and the strength increased after a time. The objects should be removed from the solution as soon as they have acquired the desired consistency, as if left too long they will become brittle. (These precautions are peculiarly necessary in the case of chromic acid.) They may be preserved till wanted in alcohol (95 per cent.). It is well to wash them out in water for twenty-four or forty-eight hours before putting them into the alcohol. I think it is frequently useful to add a little glycerin to the hardening solution; there is less brittleness and, I think, less shrinkage.

The reader's attention is called to the statements made in § 41 concerning the action of light on the alcohol containing chromic objects.

Further directions for the employment of chromic acid will be given in the special paragraphs. Chromic acid is a most powerful and rapid hardening agent (by it, you may obtain in a few days a degree of hardening that you would hardly obtain it in as many weeks with bichromate, for instance.) It has the defect of a great tendency to cause brittleness.

89. **Chromic Acid and Spirit** (URBAN PRITCHARD, *Quart. Journ. Mic. Sci.*, 1873, p. 427).—Chromic acid, 1 part; water, 20 parts; rectified spirit, 180 parts. Dissolve the chromic acid in the water first, and then add the spirit (violent action will ensue if the dry chromic acid be added directly to the spirit). The colour of the solution soon becomes brown. If, after a few days, it turns semi-gelatinous, it should be changed for fresh. From a week to ten days is required to harden such tissues as retina, cochlea, &c., for which this fluid is particularly well adapted.

90. **Chromo-osmic Acid** (MAX FLESCH.) **Chromo-aceto-osmic Acid** (FLEMMING.)—Either of these mixtures may be used for prolonged hardening, and are admirable. The weak form of Flemming's solution is the one that should generally be taken for hardening purposes. (*See* §§ 45 and 46.)

For delicate objects perhaps even better results may be obtained by means of **Chromic Acid and Platinic Chloride** (MERKEL's Solution). *See* § 52 *ante.*

91. **Picro-chromic Acid.**—This fixative may be found useful for hardening objects that are only penetrable with difficulty, some Tunicata, for instance. *See* § 51, *ante.*

92. **Osmic Acid.**—Hardly used for anything but nervous tissue. *See Neurological Methods* in Part II.

93. **Nitric Acid.**—Hardly used for anything but brain. *See* also Part II.

Salts.

94. Bichromate of Potash.—Perhaps the most important of all known hardening agents, *sensû stricto.* It hardens slowly, much more so than chromic acid, but it gives an incomparably better consistency to the tissues, and it has not the same tendency to make them brittle if the reaction be prolonged. They may remain almost indefinitely exposed to its action without much hurt.

The strength of the solutions employed is from 2 to 5 per cent. As with chromic acid, it is extremely important to begin with weak solutions and proceed gradually to stronger ones. About three weeks will be necessary for hardening a sheep's eye in solutions gradually raised from 2 to 4 per cent. Spinal cord requires from three to six weeks; a brain, at least as many months.

After hardening, the objects should be well soaked out in water before being put into alcohol. They had better be kept in the dark when in alcohol (*see* above, § 41). *If you wish to have a good stain with carmine, especially ammonia-carmine, which is admirable for portions of nervous system so hardened, you should not put the objects into alcohol at all, even for a second, until they have been stained.*

You may stain either with carmine or hæmatoxylin.

Bichromate objects have an ugly yellow colour which cannot be removed by soaking in water. It is said that it can be removed by washing for a few minutes in a 1 per cent. solution of chloral hydrate. Gierke, however, says that this treatment is prejudicial to the preservation of the tissues.

Prof. Gilson writes me that alcoholic solution of sulphurous anhydride (SO_2) is very convenient for the rapid decoloration of bichromate objects. A few drops suffice. *See* also § 41, and "Bleaching."

95. Müller's Solution.—

Bichromate of potash . . .	2—2½ parts.
Sulphate of soda . . .	1 part.
Water	100 parts.

The duration of the reaction is about the same as with the simple solution of chromic salts.

This fluid was very highly in vogue for many years, but seems lately to be much less used. I fancy that the superiority of this mixture over the simple bichromate solution is not illusory, and is due to the formation in it of a trace of free chromic acid. Fol says that for mammalian embryos, for which it has been recommended, it is worthless.

96. Erlicki's **Solution** (*Warschauer med. Zeit.*, xxii, Nos. 15 and 18).—

Bichromate of potash . . .	2·5 parts.
Sulphate of copper . . .	1·0 part.
Water	100·0 parts.

Here the addition of the cupric sulphate is intelligible. This salt is itself a hardening agent of some energy, and may well serve to reinforce the somewhat slow action of the bichromate. As a matter of fact, "Erlicki" hardens very much more rapidly than either simple bichromate or Müller's solution. A spinal cord may be hardened in it in four days at the temperature of an incubator, and in ten days at the normal temperature (Fol, *Lehrb. d. vergl. mik. Anat.*, p. 106). I believe it to be one of the best hardening agents known for voluminous objects. Human embryos of several months may be conveniently hardened in it.

Nerve-centres that have been hardened in Erlicki's fluid frequently contain dark spots with irregular prolongations, simulating ganglion-cells. These were at one time taken to be pathological formations, but they are now known to consist of precipitates formed by the action of the hardening fluid. They may be removed by washing with hot water, or with water slightly acidified with hydrochloric acid, or by treating the specimens with 0·5 per cent. chromic acid before putting them into alcohol (Tschisch, *Virchow's Arch.*, Bd. xcvii, p. 173; Edinger, *Zeit. f. wiss. Mik.*, ii, 2, p. 245; Loewenthal, *Rev. méd. de la Suisse romande*, 6me année, i, p. 20).

97. Bichromate and Platinic Mixture (Lindsay Johnson's Mixture). Latest Formula, 1895, communicated by Dr. Lindsay Johnson.—

Bichromate of potash (2·5 per cent.) .	70 parts.
Osmic acid (2 per cent.) . . .	10 ,,
Platinic chloride (1 per cent.) . .	15 ,,
Acetic or formic acid	5 ,,

It is not well to take the platinum chloride stronger than here given, as too strong solutions have a tendency to crystallize out on the tissues. Henneguy, who has worked a great deal with this reagent, and recommends it highly, says (*Leçons sur la Cellule*, Paris, Carré, 1896, p. 61) that it is well only to add the acetic or formic acid just before using, as it frequently provokes a spontaneous reduction of the osmium and platinum to such an extent that the mixture becomes quite black.

This mixture was imagined for the preliminary hardening of retina, being allowed to act for two hours only, and then being followed by final hardening in pure bichromate solution. But it has proved applicable to other structures, and gives excellent results. The function of the osmic acid in the present formula is to enhance the hardening energy of the mixture. Dr. Lindsay Johnson writes me that "it greatly reduces the length of time necessary for hardening, three days being the time from removal of the organ to its being in celloidin under dilute spirit."

"If the osmium has a tendency to blacken, this may be entirely prevented and a beautiful delicate chestnut-brown deepening towards Bartholozzi-red tint obtained by adding ten parts of 5 per cent. solution of nitrate of uranium, which forms a layer of uranium on the top of the reduced platinum and osmium (one or both)."

It has already been pointed out, No. 53A, that this mixture may be used for fixing, in some cases with the best results. HENNEGUY, *l. c.*, says it contracts the more spongy sorts of protoplasm less than mixture of FLEMMING, and that it does not produce the delusive precipitates that that mixture frequently does produce.

I am convinced that for some purposes (*e. g.* delicate researches on protoplasmic structures) it will be found well to diminish the proportion of organic acid.

98. Bichromates and Alcohol.—Mixtures of either bichromate with alcohol may be employed, and have a more rapid action than the aqueous solution. Thus HAMILTON takes for hardening brain a mixture of 1 part methylated spirits with 3 parts of solution of Müller (see the chapter on the Central Nervous System in Part II; see also KULTSCHIZKY's Mixture, *ante*, § 53). Preparations should be kept in the dark during the process of hardening in these mixtures.

99. Bichromate of Ammonia.—A review of the literature of the subject shows that this salt is in considerable favour, for what precise motive is not apparent. Its action is very similar to that of the potassium salt. Fol says that it penetrates somewhat more rapidly, and hardens somewhat more slowly. It should be employed in somewhat stronger solutions, up to 5 per cent.

100. **Neutral Chromate of Ammonia** is preferred by some anatomists. It is used in the same strength as the bichromate. Klein has recommended it for intestine, which it hardens, in 5 per cent. solution, in twenty-four hours.

Chlorides and others.

101. **Platinic Chloride Mixture** (MERKEL'S Solution).—The formula of this admirable reagent has been given above, § 52. It is an admirable hardening medium for delicate objects. Merkel states that he allowed from three to four days for the action of the fluid for the retina; for Annelids Eisig employs an immersion of three to five hours, and transfers to 70 per cent. alcohol; for small leeches Whitman finds "one hour sufficient, and transfers to 50 per cent. alcohol."

Whitman recommends, for the hardening of pelagic fish ova, a stronger mixture (due, I believe, to Eisig), viz.:

0·25 per cent. solution of platinum chloride .	1 vol.
1 per cent. solution of chromic acid . .	1 ,,

The ova to remain in it one or two days (WHITMAN, *Methods in Micro. Anat.*, p. 153).

102. **Palladium Chloride** (F. E. SCHULTZE, *Arch. mik. Anat.*, iii, 1867, p. 477).—This reagent was recommended by Schultze partly as giving to tissues a better consistency than chromic acid or Müller's solution, and partly on account of a special faculty for penetrating organs rich in connective tissue that he attributes to it. It is an impregnation reagent, staining certain elements of tissues in various tones of brown. For the somewhat lengthy details of the manner of employing it, the reader is referred to the paper quoted.

103. **Chloride of Zinc** is only employed for brain, see *post*, Part II.

104. **Picric Acid** taken alone is a weak hardening agent, little used. It should be employed in saturated solution. But it is a useful ingredient in mixtures, serving to enhance the penetrating power. See **Picro-chromic Acid**, *ante*, § 51; also Gage's **Picric Alcohol**, § 81.

105. **Acetate of Lead.**—Both the neutral acetate (sugar of lead) and the basic acetate have been used for hardening nerve tissues. ANNA KOTLAREWSKY found that nerve-cells hardened in 10 per cent. solution of sugar of lead were admirably preserved. See her "Inaug.-Diss." in *Mitth. d. naturf. Ges. Bern.*, 1887, and *Zeit. f. wiss. Mik.*, iv, 3, 1887, p. 387.

106. Iodine may be used in combination with alcohol, and render service through its great penetrating power. See the method of Betz, *post*, Part II.

107. Pyridin.—Pyridin has been recommended as a hardening agent (by A. de Souza). It is said to harden, dehydrate, and clear tissues at the same time. They may be stained after hardening by anilin dyes dissolved in the pyridin, or passed through water and stained by the usual processes. It is said to harden quickly, and to give particularly good results with brain. See *Comptes Rendus hebd. de la Soc. de Biologie*, 8 sér., t. iv, No. 35, p. 622; *Zeit. f. wiss. Mik.*, v, i, 1888, p. 65; *Journ. Roy. Mic. Soc.*, 1888, p. 1054.

108. Alcohol.—When used alone, alcohol is inferior as a hardening agent to most of the reagents discussed above; but when judicially employed to complete the action of a good fixing agent, it renders most valuable services. 90 to 95 per cent. is the most generally useful strength. Weaker alcohol, down to 70 per cent., is often indicated. Absolute alcohol is seldom advisable. You ought to begin with weak, and proceed gradually to stronger, alcohol. Large quantities of alcohol should be taken. The alcohol should be frequently changed, or the tissue should be suspended near the top of the alcohol, in order to have the tissue constantly surrounded with pure spirit (the water and colloid matters extracted from the tissue falling to the bottom of the vessel). Many weeks may be necessary for hardening large specimens. Small pieces of permeable tissue, such as mucous membrane, may be sufficiently hardened in twenty-four hours.

109. Formaldehyde (Formol, Formalin, Formalose).—This important reagent has been in part described, *ante* No. 86. Considered as a hardening agent, *sensû stricto* its most important use is for hardening nervous tissue. This will be considered in Part II.

Blum (*l. c.*, No. 86), found solutions containing 4 per cent. of formaldehyde hardened voluminous pieces of liver, kidney, stomachal mucosa, brain, &c., considerably quicker than alcohol, the preservation being excellent.

Hermann (*l. c.*, No. 86) found that such a large organ as a calf's heart was hardened by a 0·5 to 1 per cent. solution in twelve to twenty-four hours. Entire eyes are so hardened in the 1 per cent. solution in twenty-four hours that they may be cut in two with a sharp knife like an apple. Hermann found this disadvantage, that tissues hardened in formaldehyde solution suffer when they are put into alcohol for the purpose of dehydration. The paper in question contains interesting observations on the property formaldehyde has of preserving the natural colours and transparent and life-like aspect of tissues.

Blum (*Anat. Anz.*, ix, 1894, p. 229), recapitulating, says that very voluminous pieces of material are hardened quickly and without shrinkage. The tissues stain well. Cells and nuclei preserve their forms; karyokinetic figures are fixed. Mucin is not precipitated, but remains transparent; fat is not dissolved. Micro-organisms retain their specific staining reactions.

As to the *degree and kind* of hardening obtained by formaldehyde the authors are not so explicit as could be wished. As far as I can see myself, the hardening obtained is gentle and tough, giving an elastic and not a brittle consistency. It probably varies greatly with different tissues.

For prolonged hardening, considerable volumes of liquid should be taken and the liquid should be renewed from time to time. For the formaldehyde fixes itself on the tissues with which it comes in contact, deserting the solution, which thus becomes progressively weaker.

For the employment of formaldehyde in hardening nervous tissue, see Part II.

REESE LIBRARY
OF THE
UNIVERSITY
OF CALIFORNIA

CHAPTER VII.

CLEARING AGENTS.

110. Introductory Remarks.—Clearing agents are liquids one of whose functions it is to make microscopic preparations transparent by penetrating amongst the highly refracting elements of which the tissues are composed, the clearing liquids themselves having an index of refraction not greatly inferior to that of the tissues to be cleared. Hence all clearing agents are liquids of high index of refraction. The same substances have also a second function, which consists in getting rid of the alcohol in which preparations are generally preserved, and facilitating the penetration of the paraffin used for imbedding, or the balsam or other resinous medium in which preparations are, in most cases, finally mounted. Hence all of the group of bodies here called "clearing agents" must be capable of expelling alcohol from tissues, and must be at the same time solvents of Canada balsam and the other resinous mounting media. The majority of clearing agents are essential oils.

111. The Practice of Clearing.—It is important to note again, notwithstanding some repetition, the manner of employing these agents. The old plan was to take the object out of the alcohol and float it on the surface of the clearing medium in a watch-glass. This plan was faulty, because the alcohol escapes from the surface of the object into the air quicker (in most instances) than the clearing agent can get into it; hence the object must shrink. To avoid or lessen this cause of shrinkage, clearing is now generally done by the method suggested by Giesbrecht, which consists in putting the clearing medium *under* the alcohol containing the object. This is done in the following manner. Take a short glass tube, and put into it enough alcohol to contain the objects (a watch-glass will often do well, but a tube is safer). With a pipette carefully put under the alcohol a sufficient quantity of clearing medium (or carefully pour the alcohol on to the clearing medium). Then put the objects into the alcohol. They will sink down to the level of separation of the two liquids at once; and after some time they will be found to have sunk to the bottom of the clearing medium. They may then be removed by means of a pipette, or the supernatant alcohol drawn off and the preparations allowed to remain until wanted. They should not be considered to be perfectly penetrated by the clearing medium until the wavy refraction-lines caused by the mixture of the two liquids at their surface have ceased to form.

The penetration of all clearing media may be hastened by using them warm.

It frequently happens that the essential oil with which objects are being treated in a watch-glass or on a slide becomes cloudy after a short time, and fails to clear the tissues. This is owing to a combination between the essential oil and moisture, derived, I think, rather from the air than from the objects themselves. The cloudiness can usually be removed by warming (as pointed out by HATCHETT JACKSON, *Zool. Anzeig.*, 1889, p. 630), but this remedy is not always successful, for in certain states of the atmosphere the cloudiness will persist, notwithstanding continued warming. It is for this reason that I advise that clearing be done, whenever possible, in shallow well-corked tubes, under which conditions the phenomenon rarely occurs.

112. Classification of Clearing Agents.—For an account of STIEDA's experiments with essential oils, see previous editions.

NEELSEN and SCHIEFFERDECKER (*Arch. f. Anat. u. Phys.*, 1882, p. 206) examined a large series of ethereal oils (prepared by Schimmel and Co., Leipzig), with the object of finding a not too expensive substance that should combine the properties of clearing quickly alcohol preparations, *not* dissolving out anilin colours, clearing celloidin without dissolving it, not evaporating too quickly, and not having a too disagreeable smell.

Of these, the following three fulfil the conditions and can be *recommended :—Cedar-wood, Origanum, Sandal-wood.*

To these should be added the others recommended in the following paragraphs.

It would be important to possess a list of the exact indices of refraction of the substances used for clearing. I have, unfortunately, not been able to obtain sufficient information of a trustworthy nature for the compilation of such a list. Cedar oil has nearly the index of crown glass (this is true of the oil in the thick state to which it is brought by exposure to the air, not of the new, thin oil, which is less highly refractive), it therefore clears to the same extent as Canada balsam. Clove oil has a much higher index, and therefore clears more than balsam. Turpentine, bergamot oil, and creasote have much lower indices, and therefore clear less.

113. Choice of a Clearing Agent.—Special directions for clearing are given when necessary under the heads of the different organs and tissues. It will suffice here to advise the beginner to keep on his table the following:—Oil of cedar, for general use and for preparing objects for imbedding in paraffin, see "*Imbedding Methods—Paraffin*"; clove oil, for making minute dissections in cases in which it is desirable to take advantage of the property of that essence of forming very convex drops on the slide and of imparting a remarkable brittleness to soft tissues, and for much work with safranin, &c.; oil of bergamot which will clear from 90 per cent. alcohol, and which does not extract coal-tar colours; carbolic acid, for rapidly clearing very imperfectly dehydrated objects.

For special clearers for *celloidin sections* see "*Collodion (Celloidin) Imbedding Methods.*"

114. Cedar Oil (NEELSEN and SCHIEFFERDECKER, *op. cit.*, § 112).—For finest cedar-wood oil, price per kilo varies from fifteen to twenty shillings, say about sevenpence halfpenny per ounce for small quantities, or about the price of clove oil. Very thin, colour light yellow or greenish, odour slight (of cedar-wood), evaporates slowly, is not changed by light, is miscible with chloroform balsam, and with castor oil. Clears readily tissues in 95 per cent. alcohol, without shrinkage; does not extract anilin colours. Celloidin sections are cleared in five to six hours.

Cheap, but requires an inconvenient length of time for the clearing of celloidin sections.

The observer should be careful as to the quality of the cedar oil he obtains. I have examined the clearing properties of a sample obtained from the celebrated firm of Rousseau, Paris. This sample was absolutely colourless. It *totally* failed to clear absolute alcohol objects after many days. I always use the *thickened* oil as supplied for use with immersion objectives.

The authors think that a laboratory supplied with cedar oil and origanum oil is fully equipped for all possible cases (the origanum oil being used merely to take the place of cedar-wood oil for the special case of celloidin sections).

Cedar oil is very penetrating, and for this and other reasons is, in my experience, the very best of all media for preparing objects for paraffin imbedding. I find it to be less hurtful to cells and delicate tissue-structures than any other medium known to me.

115. Clove Oil.—Samples of clove oil of very different shades of colour are met with in commerce. It is frequently recommended that only the paler sorts should be employed in histology. A word of explanation is here necessary. Doubtless it is, in general, best to use a pale oil, provided it be pure, but it is not always easy to obtain a light-coloured oil that is pure. Clove oil passes very readily from yellow to brown with age, so that in choosing a colourless sample you run great risk of obtaining an adulterated sample, for clove oil is one of the most adulterated substances in commerce.

Two important properties of clove oil should be noticed here. It does not easily spread itself over the surface of a slide, but has a tendency to form very convex drops. This property makes it a very convenient medium for making minute dissections in. The second property I wish to call attention to is that of making tissues that have lain in it for some time very brittle. This brittleness is also sometimes very helpful in minute dissections.

These qualities may be counteracted if desired by mixing the clove oil with bergamot oil.

Clove oil has, I fancy, the highest index of refraction of all the usual clearing agents; it clears objects *more* than balsam. It dissolves celloidin (or collodion), and therefore should not be used for clearing sections cut in that medium, without special precautions. Notwithstanding the opinion of Schiefferdecker, I consider this to be one of the best of clearing agents, and very valuable on account of the properties to which attention has been called above. New clove oil washes out anilin colours more quickly than old. It is well to possess trustworthy samples of both new and old oil.

116. Cannel Oil.—Greatly resembles clove oil, but is in general thinner. An excellent medium, which I particularly recommend.

117. Oil of Bergamot.—SCHIEFFERDECKER (*Arch. Anat. u. Phys.*, 1882 [Anat. Abth.], p. 206) finds that this oil has many good qualities; it clears 95 per cent. alcohol preparations and celloidin preparations quickly, does not attack anilin colours, but the strong odour is disagreeable; it is as dear as oil of cloves, twice as dear as oil of origanum, and three times as dear as oil of cedar. He considers its action preferable to that of oil of cloves, but all things considered, gives the palm to cedar and origanum. I think that this is a very valuable medium, and though I do not agree with Schiefferdecker in thinking its action superior to oil of cloves, I think it should always be kept at hand.

Bergamot oil is, I believe, the least refractive of these essences, having a lower index than even oil of turpentine.

SUCHANNEK (*Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 158) says that bleached, colourless bergamot oil will not take up much water, whereas a green oil will take up as much as 10 per cent.

VAN DER STRICHT (*Arch. de Biol.*, xii, 1892, p. 741) says that bergamot oil will, with time, dissolve out the fatty granules of certain ova.

118. Oil of Origanum (NEELSEN and SCHIEFFERDECKER, *Arch. Anat. u. Phys.*, 1882, p. 204).—Price per kilo 15 marks (=15*s.*). Thin, light brown colour, odour not too strong, agreeable, does not evaporate too quickly, is not changed by light, is miscible with chloroform balsam and with castor oil. Ninety-five per cent. alcohol preparations are cleared quickly, and so are celloidin sections, without solution of the celloidin. Anilin colours are somewhat extracted.

For work with celloidin sections care should be taken to obtain *Ol. Origani Cretici* ("Spanisches Hopfenöl"), not *Ol. Orig. Gallici* (v. GIESON; see *Zeit. f. wiss. Mik.*, iv, 4, 1887, p. 482). Specimens of origanum oil vary greatly in their action on celloidin sections, and care should be taken to obtain a good sample.

Squire, in his *Methods and Formulæ*, &c., p. 81, says that origanum oil (meaning the commercial product) is nothing but oil of white thyme more or less adulterated, and that the product sold as Ol. Origani Cretici is probably oil of marjoram.

119. **Oil of Thyme.**—Fish (*Proc. Amer. Mic. Soc.*, 1893, *Zeit. f. wiss. Mik.*, xi, 4, p. 503), following Bumpus, says that for most of the purposes for which origanum oil has been recommended, oil of thyme will do just as well if not better. After one distillation of the crude oil of thyme it is of a reddish-brown colour, and is called the *red* oil of thyme; when again distilled it becomes colourless, and is distinguished as the *white* oil. The red oil is just as efficient as the white for clearing.

120. **Sandal-wood Oil** (Neelsen and Schiefferdecker, *l. c.*, § 118).—Very useful; but its high price is prohibitive.

121. **Turpentine.**—Generally used for treating sections that have been cut in paraffin, as it has the property of dissolving out the paraffin and clearing the sections at the same time; but many other reagents (see No. 126) are preferable for this purpose. If used for alcohol objects it causes considerable shrinkage, and *alters the structure of cells* more than any other clearing agent known to me, unless used in the thickened state, a method which is much liked for some purposes in Germany. Thickened turpentine ("Verhartzes Terpentinöl" of German writers) is prepared by exposing rectified turpentine in thin layers for some days to the air. All that is necessary is to pour some turpentine into a plate, cover it lightly so as to protect it from dust without excluding the air, and leave it until it has attained a syrupy consistency. Turpentine has, I believe, the lowest index of refraction of all the usual clearing agents except bergamot oil; it clears objects *less* than balsam.

122. **Carbolic Acid.**—Best used in concentrated solution in alcohol. Clears instantaneously, even very watery preparations. This is a very good medium, but it is better avoided for preparations of soft parts which it is intended to mount in balsam, as they generally shrink by exosmosis when placed in the latter medium. It is, however, a good medium for celloidin sections.

123. Gage's **Mixture** (*Proc. Amer. Soc. Micr.*, 1890, p. 120; *Journ. Roy. Mic. Soc.*, 1891, p. 418).—Carbolic acid crystals melted, 40 c.c.; oil of turpentine, 60 c.c.

124. Creasote.—Much the same properties as carbolic acid. *Beech-wood* creasote is the sort that should be preferred for many purposes,—amongst others, for clearing celloidin sections, for which it is a very good medium.

125. Anilin Oil.—This is a rather important reagent on account of its ability to clear excessively watery objects. Common anilin oil will readily clear sections from 70 per cent. alcohol, and with certain precautions (for which, see the paper of SUCHANNEK quoted below) objects may be cleared from watery media without the intervention of alcohol at all. This property renders anilin valuable in certain cases as a penetrating medium for preparing for paraffin imbedding. For ordinary work the usual commercial anilin will suffice; and it is immaterial whether it be colourless or have become brown through oxidation. For difficult work, it is well to use a perfectly anhydrous oil. For directions for preparing this see SUCHANNEK, *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 156, or the last edition of this work.

Anilin is chiefly used for clearing celloidin sections, and is sometimes found very valuable for this purpose.

126. Xylol, Benzol, Toluol, Naphtha, Chloroform.—Too volatile to be recommendable as general clearing agents, but very useful for celloidin sections or for paraffin sections. I find naphtha is quite good enough for removing paraffin from sections destined to be afterwards passed through alcohol and stained; but that it should not be used for objects destined to be passed direct into balsam, as it frequently precipitates resinous media. Of the three first mentioned liquids, benzol is the most volatile, then toluol, and xylol is the least volatile, in the proportion of 4 : 5 : 9 (SQUIRE, *Methods and Formulæ*, p. 20). Chloroform is injurious to some delicate stains.

CHAPTER VIII.

IMBEDDING METHODS—INTRODUCTION.

127. A Word on Microtomes.—It is no part of the purpose of this work to discuss instruments, yet a word on this subject may be helpful to the student. The freezing microtome so generally employed in England is less than any other form adapted to the wants of the zoologist. Very thin sections can be obtained by it more readily than with any other microtome, but they are of little use when obtained. The relations of the parts of the organs are deranged by the freezing and by the thawing, and the aqueous nature of the process prevents it from being readily applicable to the mounting of *series* of sections. The microtome of the zoologist, therefore, must be an *imbedding* microtome.

Now there are two methods of imbedding in general use—the paraffin method and the celloidin method. In the paraffin method the object is cut *dry*, with the knife set square; whilst in the celloidin method the object is usually cut *wet*, and in a softer and more elastic state than paraffin objects, and always with an obliquely-set knife. It so happens that the most precise and beautiful microtomes that have been constructed are designed in view of the paraffin method, and cannot be applied, or at all events are much less adapted, to work with celloidin objects. A thoroughly equipped laboratory should therefore possess two microtomes, one for paraffin work, and one for celloidin material, or other material that has to be cut in the wet way. If the anatomist cannot afford two instruments, he will perhaps do well not to choose one of those that are adapted only for paraffin, but to choose an all-round instrument, one that without being absolutely of the highest attainable precision in paraffin work will yet give sufficiently good results in that way, and will also cut in the wet way.

Amongst microtomes fulfilling these conditions various forms will be found almost equally convenient. Zeiss makes a good one; Schanze, of Leipzig, makes a good one; Reichert, of Vienna, makes a good one. All these are relatively cheap, and, being at the same time perfectly efficient for easy work, may be recommended. Amongst more precise instruments of this class the first place in order of date belongs to *the* THOMA *sliding microtome.* This is made in several sizes by R. Jung, Mechaniker in Heidelberg. For zoological and general histological work I recommend the *medium size* (No. 2*a* or 4), with the newest Naples object-holder and newest form of knife and knife-holder.

This instrument is described in *Journ. Roy. Mic. Soc.* (N.S.), vol. iii,

REESE LIBRARY

p. 298; the new Naples object-holder (which I consider *essential* for the zoologist) is described and figured p. 915.

The BECKER *microtome* is in many respects an improvement on the Thoma model. It is essentially on the same principle, but possesses a mechanical arrangement for moving the knife-carrier; that is, the knife-carrier is not only *guided* by a mechanical arrangement, as in the Thoma model, but is *put in motion* by mechanism. This, I think, is certainly an advantage. Another improvement is that the slides are made of glass instead of metal; this allows one to dispense with the use of oil to the slides, which in the Thoma model gives rise to inequality in the thickness of sections. A minor point is that the instrument is somewhat cheaper than the Thoma form. It is made by Aug. Becker, Göttingen. Descriptions of two forms (Spengel and Schiefferdecker) will be found in *Journ. Roy. Mic. Soc.*, 1886, pp. 884 and 1084. The Naples object-holder can be fitted to the Becker microtome.

The instruments above described are "all-round" microtomes; by which is meant that they may be used either with a square-set knife or an obliquely-set knife, and will cut either celloidin sections or frozen preparations (if a freezing apparatus be added to them) just as well as paraffin sections. They will not, according to my experience, cut series of paraffin sections with anything like the same infallible regularity, certainly not with the same rapidity as the instruments next to be mentioned. But they give excellent results, and in view of their adaptability to celloidin or other semi-soft preparations, I think that one of them, the Becker by preference, should be the instrument chosen by the worker who desires not to be entirely confined to the paraffin method, and who cannot conveniently possess more than one microtome.

All the instruments mentioned hitherto are *sliding microtomes*, that is instruments in which the object to be cut is a fixture during cutting, and the knife is moved on a slide and is only attached to its holder at one end. This arrangement will not allow the highest possible accuracy to be obtained with paraffin objects or any other hard objects. For with hard objects the knife is free to tilt slightly on meeting the object, instead of cutting its way through it. This defect is fatal to the attainment of perfectly cut series of sections of equal thickness throughout. For the highest class of work it is necessary to employ a microtome constructed on the opposite principle, namely one in which the knife is a fixture, and fixed at both ends as near as possible to the cutting point; the object being moved against it. The following instruments are constructed on this principle, and for accurate cutting of paraffin sections are undoubtedly superior to any sliding microtome; they also work incomparably quicker.

The *Cambridge rocking microtome* (furnished by the Cambridge Scientific Instrument Company, Carlyle Road, Cambridge, price £4 4*s*., or by Messrs. Swift and Son, or by Jung) is only adapted for cutting paraffin sections (Mr. Swift has shown me an arrangement for inclining the knife so as to give it the position required for cutting celloidin; but I feel pretty sure that this will prove a failure in practice). This instrument is extremely simple and extremely rapid, and, what is more important, cuts more level series of sections than any other microtome I am personally acquainted with. It should be fitted with an adjustable object-holder, allowing of precise orientation of the object. This, I believe, has been done in the newest instruments. Or the object-holder of Henneguy and Vignal (*Compt. Rend. Soc. Biol.*, 1885, p. 647), may be added to it. (This, as well as the entire instrument, is manufactured in France by Dumaige, 24, Rue Saint-Merri, Paris, or Messrs. Swift on request will furnish such an arrangement, or it may be obtained, with or without the entire instrument, from Jung, of Heidelberg). See also in *Zeit. f. wiss. Mik.*, iv, 4, 1887, p. 465, the description of an object-holder adapted to the rocking microtome by HASSELAER; further, the price list of JUNG; also a paper in *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 165.

It has been objected to this instrument by Schiefferdecker (see *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 171, a description and criticism of the instrument as made by Jung) that it does not cut plane sections, but sections having the form of segments of a cylinder. This is true; but it does not therefore follow, as Schiefferdecker concludes, that the instrument is inapplicable to many morphological purposes, and especially to embryological research. In practice, the slight deviation of the sections from a plane figure is found to be quite inappreciable, and therefore unimportant. And if I rightly understand, this slight defect has been overcome in the new model (1895).

REESE LIBRARY OF THE UNIVERSITY OF CALIFORNIA

Rather more costly (£8 15s.) is the MINOT microtome made by E. Zimmermann, Mechaniker, 21, Emilien Strasse, Leipzig. A description and figures of this instrument will be found in *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 176, or in *Journ. Roy. Mic. Soc.*, 1889, p. 143. It is worked on the sewing-machine principle: the knife is fixed as in the Cambridge instrument, and the object is made to impinge on it by means of a rotary motion given to a wheel by the hand, and converted by a crank and slide lever into a vertical one given to a slide carrying the object. This microtome cuts with very great rapidity, and those who have worked with it speak very highly of it. It is said that owing to the construction of the slide, which is subject to uncompensated wear and tear, its work is liable to fail in accuracy. The object-holder does not appear to be so scientifically constructed as the Naples one. Like the Cambridge instrument, this microtome is only adapted for paraffin work, and for this reason and the others stated above I do not feel satisfied that it should be preferred to the Becker or Thoma by those who have to be content with a single instrument.

The most beautiful of all these instruments is the REINHOLD-GILTAY. It is constructed on essentially the same principle as the Minot, but the detail has been further elaborated, with the result of obtaining an instrument that is at the same time more precise in operation and more resistant to wear and tear, all working parts being compensated throughout. I have not had the advantage of working with this machine myself, but I have seen it in operation, and can testify that with a common razor I have seen it cut perfectly continuous ribbons of sections of one micron in thickness, from a block of paraffin half an inch square. The sections were all of them entire and without any defect, further than that some of them were somewhat compressed, a fault which is, of course, the expression of insufficient keenness in the knife, not of any want of accuracy in the machine.

The Reinhold-Giltay has an arrangement for allowing the cutting of collodion material. I am unable to say whether this is a success. It is made by J. W. Giltay, Delft, and costs about twenty pounds. A description will be found in *Zeit. f. wiss. Mik.*, ix, 4, 1893, p. 445, and in *Journ. Roy. Mic. Soc.*, 1893, p. 706.

128. Imbedding Methods.—The processes known as Imbedding Methods are employed for a twofold end. Firstly, they enable us to surround an object, too small or too delicate to be firmly held by the fingers or by any instrument, with some plastic substance that will support it on all sides with firmness but without injurious pressure, so that by cutting sections through the composite body thus formed, the included object may be cut into sufficiently thin slices without distortion. Secondly, they enable us to fill out with the imbedding mass the natural cavities of the object, so that their lining membranes or other structures contained in them may be duly cut *in situ;* and, further, they enable us to surround with the supporting mass not only each individual organ or part of any organ that may be present in the interior of the object, but each separate cell or other anatomical element, thus giving to the tissues a consistency they could not otherwise possess, and ensuring that in the thin slices cut from the mass all the details of structure will precisely retain their natural relations of position. Such a process of imbedding is at the same time practically a process of hardening in so far as it gives to tissues a degree of firmness that could otherwise only be obtained by the employment of chemical processes such as prolonged treatment with chromic acid and the like.

These ends are usually attained in one of two ways. Either the object to be imbedded is saturated by soaking with some material that is liquid while warm and solid when cold, which is the principle of the processes here called *Fusion Imbedding Methods;* or the object is saturated with some substance which whilst in solution is sufficiently fluid to penetrate the object to be imbedded, whilst at the same time, after the evaporation or removal by other means of its solvent, it acquires and imparts to the imbedded object sufficient firmness for the purpose of cutting. The collodion process sufficiently exemplifies this principle. If a piece of soft tissue be dehydrated, and soaked first in ether and then in collodion, and if the ether contained in the collodion be allowed slowly to evaporate, the tissue and surrounding mass of collodion will acquire a consistency such as to admit of thin sections being cut from them. The methods founded on this principle are here called *Evaporation Imbedding Methods.*

In any of these cases the material used for imbedding is technically termed an "imbedding mass"—*Einbettungsmasse —masse d'inclusion.* Imbedding methods are spoken of by French writers as *méthodes d'inclusion,* or *méthodes d'enrobage.*

As before stated, the method most generally employed, and the one which may be considered the normal anatomical method, is the paraffin method.

129. Imbedding Manipulations.—Before proceeding to describe in detail the more important imbedding methods, it is necessary to give an account of the manipulations of the process of imbedding in general.

Imbedding in a melted mass such as paraffin is performed in one of the following ways. A little tray or box or thimble is made out of paper, some melted mass is poured into it; at the moment when the mass has cooled so far as to have a consistency that will not allow the object to sink to the bottom, the object is placed on its surface, and more melted mass poured on until the object is enclosed. Or the paper tray being placed on cork, the object may be fixed in position in it whilst empty by means of pins and the tray filled with melted mass at one pour. The pins are removed when the mass is cold.

In either case, when the mass is cold the paper is removed from it before cutting.

To make **paper trays**, proceed as follows. Take a piece of stout paper or thin cardboard, of the shape of the annexed figure (Fig. 1); thin (foreign) post-cards do very well indeed. Fold it along the lines *a a′* and *b b′*, then along *c c′* and *d d′*, taking care to fold always the same way. Then make the folds *A A′, B B′, C C′, D D′,* still folding the same way. To do this you apply *A c* against *A a,* and pinch out the line *A A′,* and so on for the remaining angles. This done, you have an imperfect tray with dogs' ears at the angles. To finish it, turn the dogs' ears round against the ends of the box, turn down outside the projecting flaps that remain, and pinch them down. A well-made post-card tray will last through several imbeddings, and will generally work better after having been used than when new.

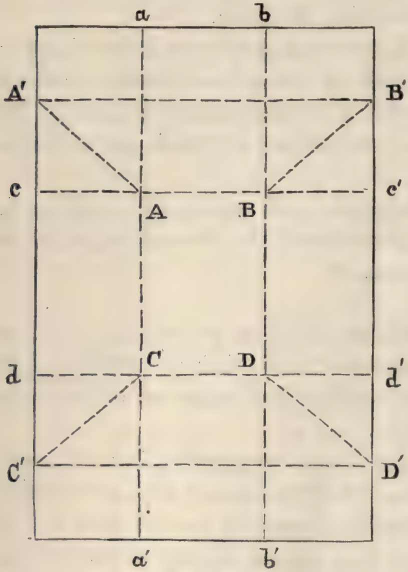

FIG. 1.

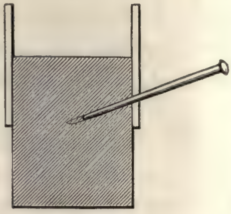

FIG. 2.

To make **paper thimbles**, take a good cork, twist a strip of paper several times round it so as to make a projecting collar, and stick a pin through the bottom of the paper into the cork. For work with fluid masses, such as celloidin, the cork may be leaded at the bottom to prevent it from floating when the whole is thrown into spirit or other liquor for hardening (Fig. 2).

LEUCKHART'S **Imbedding Boxes** are made of two pieces of type-metal (Fig. 3). Each of these pieces has the form of a carpenter's "square" with the end of the shorter arm triangularly enlarged outwards. The box is constructed by placing the two pieces together on a plate of glass which has been wetted with glycerin and gently warmed. The area of the box will evidently vary according to the position given to the pieces, but the height can be varied only by using different sets of pieces. In

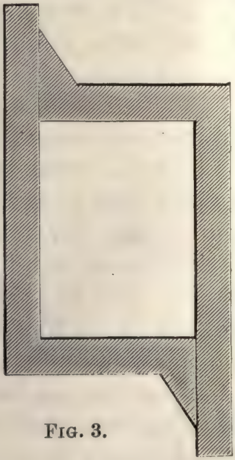

FIG. 3.

such a box the paraffin may be kept in a liquid state by warming now and then over a spirit lamp, and small objects be placed in any desired position under the microscope (*Journ. Roy. Mic. Soc.* [N.S.], ii, p. 880).

Selenka has described and figured a simple but perhaps more efficacious apparatus having the same object. It consists of a glass tube, through which a stream of warm water may be passed and changed for cold as desired, the object being placed in a depression in the middle of the tube (see *Zool. Anz.*, 1885, p. 419). A modification of this method is described by Andrews in *Amer. Natural.*, 1887, p. 101; cf. *Zeit. f. wiss. Mik.*, iv, 3, 1887, p. 375.

For **small paraffin objects** the following procedure is very useful. The object is removed from the paraffin solution, the superfluous fluid is removed by means of blotting-paper, and the object placed on a cylinder of paraffin. A piece of stout iron wire is now heated in the flame of a spirit lamp, and with it a hole is melted in the end of the cylinder; the specimen is pushed into the melted paraffin, and placed in any desired position. The advantages of the method lie in the quickness and certainty with which it can be performed.

I strongly recommend the reader not to neglect this simple method, which is capable of sometimes rendering services which no other method can. Those who have to do work with objects so small that their position can only be made out with the aid of a powerful lens ought to know how to arrange an object with a heated needle under a dissecting microscope, or on the object-carrier of the microtome.

[In the first edition this procedure was attributed to Kingsley. It appears to have been first published by Born, see "Die Plattenmodellirmethode," in *Arch. f. mik. Anat.*, 1883, p. 591.]

There remains **the watch-glass method**. Melt paraffin in a watch-glass, and throw the object into it; or place the object in the watch-glass, add solid paraffin, and heat. After the mass has hardened, cut out a block containing the object (*this is of course applicable to other masses,* such as celloidin). If paraffin be used you may, instead of cutting out a block, turn out the whole mass of paraffin by simply warming rapidly the bottom of the glass, but I find it is far safer to cut out a block. To facilitate the removal of the mass some persons lubricate the watch-glass before pouring in the mass. To do this a drop of glycerin should be smeared over it and wiped off with a cloth until hardly a trace of it remains. But this is not necessary.

As regards the merits of the watch-glass process, I wish to say that, as regards *small objects* at all events, I consider it *the very best process of any.*

130. Choice of a Method.—Amongst the very various methods of imbedding that have been proposed two are pre-eminently important—the paraffin method for small objects, and the celloidin or collodion method for large objects.

The subject of the respective merits of paraffin and celloidin still affords matter for discussion to some persons. The case, however, seems to be a very simple one. Celloidin does not afford by a long way the thinnest sections that are obtainable with small objects. For such objects it is, therefore, not equal to the demands made by modern minute anatomy, and paraffin must be taken. On the other hand, paraffin (as at present employed) will only cut very thin sections with fairly small objects; with objects of much over half an inch in diameter you cannot get with paraffin thinner sections than you can with celloidin; and if you try to cut in paraffin objects of still greater size, say an inch and upwards, it will frequently happen that you will not get perfect sections at all, blocks of paraffin of this size having a tendency to split under the impact of the knife; so that for very large objects celloidin gives better results.

I have not been able to satisfy myself that the preservation of the tissues is better in celloidin sections than in paraffin sections; so that—convenience apart—the case remains as above stated,—paraffin for small sections, celloidin for large ones.

To this must be added aqueous masses, such as gum or gelatin, for very special cases.

It will be the purpose of the next chapter to describe the paraffin method, and to mention some other masses that can be employed in a similar manner, the celloidin method and the other methods that remain to be described being treated of in the following Chapter.

CHAPTER IX.

IMBEDDING METHODS—PARAFFIN AND OTHER FUSION MASSES.

131. Penetration or Clearing.—The first stage of the paraffin method consists in the penetration or infiltration of the object by some substance which is a solvent of paraffin. The process may be called a clearing process, since the chief substances used for infiltration are also "clearing" agents.

The process of penetration or clearing should be carefully performed with well-dehydrated objects in the manner described in § 111.

Penetration liquids being merely liquids that are, on the one hand, miscible with alcohol, and on the other hand good solvents of paraffin, are as numerous as could be wished. Amongst them may be mentioned essence of turpentine, clove oil, bergamot oil, creasote, benzol, xylol, toluol, naphtha, oil of cedar wood, chloroform, and anilin oil.

Turpentine penetrates well, and mixes readily with paraffin. I do not, however, recommend it, because in my experience it is of all others the clearing agent that is the most hurtful to delicate structures.

Clove oil penetrates well, and preserves delicate structures well; but it mixes very slowly with paraffin, and quickly renders tissues brittle.

Benzin has been recommended by Brass (*Zeit. f. wiss. Mik.*, ii, 1885, p. 301).

Toluol (or toluen) has been recommended by Holl (*Zool. Anz.*, 1885, p. 223).

Naphtha has been recommended by Webster (*Journ. Anat. and Physiol.*, xxv, 1891, p. 278). For large specimens it has the advantage of being very cheap. Dr. Webster writes me that a quality known as "Persian naphtha" is best for fine work, but the common pure naphtha is sufficient for ordinary work.

Field and Martin (*Zeit. f. Wiss. Mik.*, xi, 1, 1894, p. 10) recommend a light petroleum known as "petroleum-æther."

Xylol is said by M. Heidenhain (*Kern und Protoplasma*, p. 114) to be a cause of shrinkage in cells; he employs oil of bergamot.

Chloroform mixes well with paraffin, and after evaporation in a paraffin bath (in the manner described in the next paragraph) leaves behind a pure and very homogeneous paraffin, having but little tendency to crystallise. But it is deficient in penetrating power, so that it requires an excessive length of time for clearing objects of any size; and it must be very thoroughly got rid of by evaporation in the paraffin bath, or by successive baths of paraffin, as if the least trace of it remains in the paraffin used for cutting it will make it soft. The process of removal requires a very long time, in some cases days. Chloroform ought therefore to be reserved for small and easily penetrable objects.

Cedar-wood oil is, according to my continued experience, for the reasons stated by me in *Zool. Anz.*, 1885, p. 563, in general the *very best* clearing agent for paraffin imbedding. It penetrates rapidly, preserves delicate structure better than any clearing agent known to me, does not make tissues brittle, even though they may be kept for weeks or months in it, and has the great advantage that if it be not entirely removed from the tissues in the paraffin bath it will not seriously impair the cutting consistency of the mass; indeed, I fancy it sometimes improves it by rendering it less brittle. I always use the *thickened* oil as supplied for use with immersion objectives.

In some difficult cases anilin oil is indicated (see § 125).

132. The Paraffin Bath.—The objects having been duly "penetrated" or "cleared," the next step is to substitute melted paraffin for the penetrating or clearing medium.

Some authors lay great stress on the necessity of making the passage from the clearing agent to the paraffin as gradual as possible, by means of successive baths of mixtures of clearing agent and paraffin kept melted at a low temperature, say 35° C. With oil of cedar or toluol, at all events, this is not necessary. All that is necessary is to bring the objects into melted paraffin kept just at its melting-point, and keep them there till they are thoroughly saturated; the paraffin being changed once or twice for fresh only if the objects are sufficiently voluminous to have brought over with them a notable quantity of clearing agent.

The practice of giving successive baths first of soft and then of hard paraffin appears to me *entirely illusory*.

It is important to keep the paraffin *dry*—that is, protected from vapour of water during the bath.

It is still more important to keep it as nearly as possible at melting-point. If it be heated for some time to a point much over its normal melting-point, *the melting-point will rise*, and you will end by having a harder paraffin than you set out with. And as regards the preservation of tissues, of course the less they are heated the better.

The *duration of the bath* must, of course, vary according to the size and nature of the object. An embryo of the size of a pea ought to be thoroughly saturated after an hour's bath, or often less, if cedar oil has been used for clearing. In any case, the preparations should be *cooled* (see below, § 135) *as soon as saturated*. If left for many hours in a warm bath, as is sometimes done, delicate structures may be seriously injured. Indeed, the great point to be attended to in paraffin work of the finer order is to minimise the action of heat. It is therefore important both to employ a paraffin of the lowest melting-point that will give good sections (see below, § 142), and to abbreviate the warm bath as much as possible.

If chloroform or other volatile agent be taken, choice may be made of two methods: either, as in Giesbrecht's method, the chloroform containing the object is heated to the melting-point of the paraffin, and the paraffin gradually added, and the mass kept at the melting-point of the pure paraffin until all the chloroform is driven off; or, as in Bütschli's method, the objects are simply passed direct from chloroform into a solution of paraffin in chloroform, in which they remain until thoroughly impregnated (half to one hour), and which is then evaporated at the melting-point of the paraffin. Bütschli recommends a paraffin solution melting at 35°. (Such a solution is made of about equal parts of chloroform and paraffin of 50° melting-point.) Or, in the case of larger objects, instead of evaporating the chloroform (which is often a very long process, as the chloroform must be *completely* driven off, or the mass will remain too soft for cutting), Bütschli simply transfers them from the bath of paraffin solution to a bath of pure paraffin.

Giesbrecht's method (*Zool. Anz.*, 1881, p. 484), more fully stated, is as follows:

Objects to be imbedded are saturated with absolute alcohol and then brought into chloroform (to which a little sulphuric ether has been added if necessary, in order to prevent the objects from floating). As soon as the objects are saturated with the chloroform, the chloroform and objects are gradually warmed up to the melting-point of the paraffin employed, and during the warming small pieces of paraffin are by degrees added to the chloroform. So soon as it is seen that no more bubbles are given off from the objects, the addition of paraffin may cease, for that is a sign that the paraffin has entirely displaced the chloroform in the objects. This displacement having been a *gradual* one, the risk of shrinkage of the tissues is reduced to a minimum.

133. Stoves and Water-baths.—It is important that the paraffin should not be exposed to a moist atmosphere whilst it is in the liquid state. If a water-bath be used for keeping it at the required temperature, provision should be made for protecting the paraffin from the steam of the heated water.

A very convenient apparatus for this purpose is that of Paul Mayer, which will be found described at p. 146 of *Journ. Roy. Mic. Soc.*, 1883. It may be procured from the Zoological Station at Naples (address—"Direzione della Stazione Zoologica, Napoli"), or from M. Paul Rousseau, 24, rue des Fossés-St.-Jacques, Paris. See also *Amer. Natural.*, 1886, p. 910; and *Journ. Roy. Mic. Soc.*, 1887, p. 167.

Other similar forms of paraffin-heating apparatus are described in several places in the same journal, as also in *Zeit. f. wiss. Mik.*

But whenever the worker has gas at his disposition, it will be found preferable to employ a regulating stove or thermostat. I recommend the form described in FOL's *Lehrbuch*, p. 121. Other descriptions of similar apparatus will be found also in the above-named journals.

Amongst apparatus arranged for heating by means of petroleum or similar combustibles other than gas may be mentioned the stove manufactured and sold by F. SANTORIUS, Göttingen (*Zeit. f. wiss. Mik.*, x, 2, 1893, p. 161), and that of ALTMANN (*ibid.*, p. 221, cf. *Centralb. f. Bakteriol.*, xii, 1892, p. 654).

134. Imbedding IN VACUO.—There are objects which, on account of their consistency or their size, cannot be penetrated by paraffin in the ordinary way, even after hours or days in the bath. For such objects the method of imbedding in a vacuum renders the greatest service. It not only ensures complete penetration in a very short time—a few minutes—but it has the further advantage of *preventing any falling in of the tissues* such as may easily happen with objects possessing internal cavities if it be attempted to imbed them in the ordinary way.

The principle of this method is that the objects are put through the paraffin-bath *in vacuo*. In practice this may be realised by means of any arrangement that will allow of maintaining paraffin at the necessary temperature for keeping it fluid under a vacuum.

The apparatus of HOFFMANN will be found described and figured at p. 230 of *Zool. Anz.*, 1884. In this arrangement the vacuum is produced by means of a pneumatic water aspiration pump, the vessel containing the paraffin being placed in a desiccator heated by a water-bath and furnished with a tube that brings it into communication with the suction apparatus. This arrangement is very efficacious and very simple if the laboratory possesses a supply of water under sufficient pressure.

In order to obtain the requisite vacuum without the aid of water under pressure, a simple little apparatus has been designed by FRANCOTTE (*Bull. Soc. Belg. Micr.*, 1884, p. 45). In this the vacuum is produced by the condensation of steam.

FOL (*Lehrb.*, p. 121) employs the vacuum apparatus of Hoffmann, but simplifies the arrangement for containing the paraffin. The paraffin is contained in a stout test-tube furnished with a rubber stopper traversed by a tube that puts it into communication with the pump. The lower end of the test-tube dips into a water-bath. You pump out the air once or twice, wait a few minutes to make sure that no more bubbles rise, then let the air in, turn out the object with the paraffin (which by this time will have become abnormally hard), and re-imbed in fresh paraffin.

See also a paper by PRINGLE, in *Journ. Path. and Bacteriol.*, 1892, p. 117; or *Journ. Roy. Mic. Soc.*, 1892, pp. 893.

135. Imbedding and Cooling.—As soon as the objects are thoroughly saturated with paraffin they should be imbedded by one of the methods given above (§ 129). *If the watch-glass method be followed* the paraffin-bath will naturally have been given in the watch-glass used for imbedding, and *no special imbedding manipulation will be necessary.* In any case the important point now to be attended to is that *the paraffin be cooled as rapidly as possible.* The object of this is to prevent crystallisation of the paraffin, which may happen if it be allowed to cool slowly, and to get as homogeneous a mass as possible.

Very small objects may be taken out of the paraffin with a needle or small spatula, and put to cool on a block of glass, then imbedded in position for cutting on a cone of paraffin by means of a heated needle in the manner described above

(§ 129). In the use of the needle it should be noted that it is important *to melt as little paraffin as possible at one time,* in order that that which is melted may cool again as rapidly as possible.

If the watch-glass method be adopted, float the watch-glass with the paraffin and objects on to cold water. Do not let it sink till all the paraffin has solidified. When cool, cut out blocks containing the objects; do this with a *slightly* warmed scalpel.

If paper trays be taken, cool them on water, holding them above the surface with only the bottom immersed until all the paraffin has solidified, as if you let them go to the bottom at once you will probably get cavities filled with water formed in your paraffin. Or you may put them to cool on a block of cold metal or stone.

SELENKA recommends cooling the mass by passing a stream of cold water through the imbedding tube described above (§ 129).

The objects having been mounted on the carrier of the microtome in position for cutting, pare the blocks square to the knife, and sufficiently close down to the objects, and go round them with a lens. If any bubbles or cavities or opaque spots be present, prick with a heated needle till all is smooth and homogeneous. Minutes spent in this way are well invested.

It is said by some workers that it is well to cut within a few hours of imbedding if the structure be at all delicate, as paraffin may continue to crystallize slowly to a certain extent even after rapid cooling. But this danger is very greatly diminished if the mass have been properly cooled. And according to my experience the damage likely to arise from the crystallisation of the paraffin has been greatly exaggerated. As stated in § 3, I find no better medium for the preservation of tissues than paraffin.

136. Orientation.—The above-described manipulations of definitive imbedding are in most cases sufficient. But it may be desirable to have the object fixed in the cooled block in a more precisely-arranged position, and, above all, in a more precisely-marked position, than is possible by these simple methods. Here is a method due to PATTEN (*Zeit. f. wiss. Mik.*, xi, 1, 1894, p. 13), which is especially useful when one desires to orient accurately large numbers of small and similar objects. You get some writing paper of the sort that is made with two sets of raised parallel lines running at right angles to each other (according to WOODWORTH, see below, this is known as "linen cloth paper"). Small strips are cut from this, and at suitable intervals along them small drops of a mixture of collodion and clove oil, of about the consistency of thick honey, are arranged close together along one of the ribs that run lengthwise. The objects to be imbedded are cleared in olive oil or oil of bergamot—not turpentine. They are taken one by one on the point of a knife, and after the excess of oil has been drawn off are transferred each to a drop of the collodion mixture. They may be adjusted therein under the dissecting microscope, and will stay in any required position. When half a dozen or more objects have been oriented in reference to the cross lines (which are to be parallel to the section planes) the whole thing is placed in turpentine. This washes out the clove oil and fixes the objects very firmly to the paper. The paper with the attached objects is now passed through the bath of paraffin and imbedded in the usual way. After cooling on water the block is trimmed and the paper peeled off, leaving the objects in the paraffin close to the under surface of the block. This surface is now seen to be marked by the orienting lines of the ribbed paper, and also by any record numbers which may before imbedding have been written with a soft pencil on the paper.

A somewhat more complicated form of this process has been described by WOODWORTH, *Bull. Mus. Comp. Zool.*, xxxviii, 1893, p. 45.

A similar process has also been described by FIELD and MARTIN in *Zeit. f. wiss. Mik.*, xi, i, 1894, p. 11, small strips of gelatin being used instead of paper.

137. Cutting and Section-stretching.—Paraffin sections are cut *dry*—that is, with a knife not moistened with alcohol or other liquid. By this means better sections are obtained, but a difficulty generally arises owing to the tendency of sections so cut to curl up on the blade of the knife. It is sometimes difficult by any means to unroll a thin section that has curled. To prevent sections from rolling, the following points should be attended to.

First and foremost, the paraffin *must not be too hard*, but must be taken of a melting-point suitable to the temperature of the laboratory (for the winter season, the temperature of the laboratory being between 15° and 17° C., a paraffin melting at about 45° C. should be taken; for hot summer weather, laboratory at 22° C., a paraffin of 48° C. melting-point; but see further, § 142).

The exact degree of hardness necessary must be determined by experiment. If, after cutting has begun, the paraffin be found to be too hard, it may be softened by the following simple expedient (FOL, *Lehrbuch. d. vergl. mikr. Anat.*, p. 123):—A lamp provided with a parabolic reflector is set up near the microtome in such a position that the heat-rays of the flame are thrown by the reflector on to the imbedded object. The right temperature is obtained by adjusting the distance of the lamp. This is not a mere theoretical fancy, but a very practical hint. I find that a reflector is not necessary, and that a mere spirit-lamp set up near the object will sometimes bring the paraffin to the right consistency in a few minutes.

If, on the contrary, the paraffin be found too soft, it may be hardened by exposing it to the cooling influence of a lump of ice placed in the focus of a similar reflector.

It is often sufficient to moderate the temperature of the room by opening or closing the window, stirring the fire, setting up a screen, or the like.

Secondly, the knife should be set square, for the oblique position produces rolling, and the more the knife is oblique the more do the sections roll.

Thirdly, it is better to cut ribbons than disconnected sections; ribbons of sections will often cut perfectly flat, even when the same mass will only give rolled sections if cut disconnectedly.

Special masses having less tendency to roll than pure paraffin have been proposed. Thus a mass composed of four parts of hard paraffin and one of vaselin has been recommended. I recommend, however, that all such mixtures be avoided.

Mechanical means may be employed. The simplest of these and perhaps the best is as follows:

During the cutting the edge of the section that begins to curl is caught and held down on the blade of the knife by means of a small camel-hair brush with a flat point, or by a small spatula made by running a piece of paper on to the back of a scalpel. Or the section is held down by means of an instrument called a "section-stretcher." This consists essentially of a little metallic roller suspended over the object to be cut in such a way as to rest on its free surface with a pressure that can be delicately regulated so as to be sufficient to keep the section flat without in any way hindering the knife from gliding beneath it.

According to *Ref. Handbook Med. Sci.*, Supp., p. 441, a very simple section-smoother may be made as follows:—The head and point are cut off an ordinary pin, which is then fixed parallel to the edge of the knife by pressing its ends down into two small pellets of beeswax to the proper depth.

Amongst the more complicated devices that have been imagined, that of BORN (*Zeit. f. wiss. Mik.*, x, 2, 1893, p. 157, or *Journ. Roy. Mic. Soc.*, 1894, p. 132) appears to me to be the most recommendable. It must be allowed that all these instruments are difficult to use, and that if they are not perfectly adjusted they may easily injure the sections.

See the descriptions of various forms of section-stretchers, *Zool. Anzeig.*, vol. vi, 1883, p. 100 (SCHULTZE); *Mitth. Zool. Stat. Neapel*, iv, 1883, p. 429 (MAYER, ANDRES, and GIESBRECHT); *Arch. f. mik. Anat.*, xxiii, 1884, p. 537 (DECKER); *Bull. Soc. Belge Mic.*, x, 1883, p. 55 (FRANCOTTE); *The Microscope*, February, 1884 (GAGE and SMITH); WHITMAN'S *Meth. in Mic. Anat.*, 1885, p. 91; *Zeit. f. wiss. Mik.*, iv, 2, 1887, p. 218 (STRASSER); as well as *Journ. Roy. Mic. Soc.*, iii, pp. 450, 916, and other places.

Another plan is to allow the sections to roll, but to control the rolling. To this end, the block of paraffin is pared to the shape of a wedge five or six times as long as broad, the object being contained in the broad part, and the edge turned towards the knife. The sections are allowed to roll and come off as coils, the section of the object lying in the outermost coil, which will be found to be a very open one—indeed, very nearly flat. Lay the coil on a slide with this end downwards, warm gently, and the part containing the object will unroll completely and lie quite flat.

Minute examination of paraffin sections sometimes reveals certain distortions and dislocations or even ruptures of delicate elements. I have often noticed that in certain regions of my sections all the karyokinetic figures are drawn up to one side (always the same side) of the nucleus, leaving the rest of the nucleus empty and vacuolar in appearance. The achromatic fibrils of the division spindle are frequently ruptured, and I have not rarely found isolated chromosomes lying far from the nucleus in the body of cells, or even outside the cells themselves. These phenomena have generally been ascribed to "shrinkage" caused by the action of the fixing agents or the processes of dehydration or imbedding. HEIDENHAIN (*Ueber Kern u. Protoplasma*, in *Festschr. Herrn Geheimr. A. v. Kölliker gewidm.*, 1892) thinks that they are often caused by *excessive tilt* of the under surface of the microtome knife. If this be found to be the case, the knife should be readjusted by means of a piece of cardboard placed in the jaws of the clamp. I would suggest that another cause of these defects is to be found in imperfection of the edge of the knife. If the knife be blunt, or have a rounded or curled edged caused by untrue honing or stropping, it will of course act in respect to minute structures as a plough rather than a cutting instrument, and thus produce the appearances described.

Devices for heating or for cooling the knife, with a view to the improvement of cutting, have been described; see VAN WALSEM in *Zeit. f. wiss. Mik.*, xi, 2, 1894, p. 218. I have myself sometimes found it advantageous to warm the knife.

138. Chain or Ribbon Section-cutting.—If a series of paraffin sections be cut in succession and not removed from the knife one by one as cut, but allowed to lie undisturbed on the blade, it not unfrequently happens that they adhere to one another by the edges so as to form a chain which may be taken up and transferred to a slide without breaking up, thus greatly lightening the labour of mounting a series. The following appear to me to be the factors necessary for the production of a chain.

Firstly, the paraffin must be of a *melting-point* having a certain relation to the temperature of the laboratory. *Small* sections can always be made to chain when cut from a good paraffin of 45° C. *melting-point in a room in which the thermometer stands at* 16° *to* 17° C. (The temperatures quoted apply to the case of rooms heated by an open fire, and probably would not apply to the case of rooms heated by closed stoves, such as are usual in Germany.) At 15° C. the paraffin will be found a trifle hard. At 22° C. the proper melting-point of the paraffin will probably be found at about 48° C., but my observations at these temperatures are less extended. Secondly, the *knife should be set square.* Thirdly, the block of paraffin should be pared down very close to the object and should be cut so as to present a straight edge parallel to the knife edge; and the opposite edge should also be parallel to this. Fourthly, the sections ought to be *cut rapidly,* with the swiftest strokes that can be produced. For it is the sharp impact of the knife that slightly heats, and therefore slightly softens the near edge of the paraffin, and thus causes the sections to cohere. It is by no means necessary for this purpose to have recourse to special mechanical contrivances, as in the so-called ribbon microtomes. The Thoma microtome well flooded with oil is sufficient. But the automatic microtomes are certainly most advantageous for this purpose, and amongst them the Cambridge Rocking Microtome, the Reinhold-Giltay, and the Minot, may be quoted as giving admirable results.

Various plans, such as coating the edges of the paraffin with softer paraffin, or with Canada balsam, or the employment of specially prepared paraffin, have been recommended, with the idea that they help the sections to stick. None of these devices is necessary. For the prepared paraffin of Spee, Brass, and Foettinger, see below, § 143.

It sometimes, though rarely, happens that the ribbon becomes electrified during the cutting, and twists and curls about in the air in a most fantastic manner. It may be got flat by warming slightly; but there is no known means of preventing the electrification.

139. Collodionisation of Sections.—Some objects are by nature so brittle that, notwithstanding all precautions taken in imbedding and previous preparation, they break or crumble before the knife, or furnish sections so friable that it is impossible to mount them in the ordinary way without some impairment of their integrity. Ova are frequently in this case. The remedy for this state of things consists in covering the exposed surface of the object just before cutting each section with a thin layer of colloidin, which serves to hold together the loose parts of even the most fragile sections in a wonderfully efficacious way; and the same treatment applied to tissues which are not specially fragile will enable the operator to cut sections considerably thinner than can be obtained in the usual way. Bütschli has obtained in this manner sections of less than 1 μ in thickness.

The primitive form of the process was to place a drop of collodion on the free surface of each section just before cutting it. But this practice has two defects; the quantity of collodion employed sensibly softens the paraffin, and the thick layer of collodion when dry causes the sections to roll.

Mark (*Amer. Natural.*, 1885, p. 628; cf. *Journ. Roy. Mic. Soc.*, 1885, p. 738) gives the following directions:

"Have ready a little very fluid collodion in a small bottle, through the cork of which passes a small camel-hair brush, which just dips into the collodion with its tip. The collodion should be of such a consistency that when applied in a thin layer to a surface of paraffin it dries in two or three seconds without leaving a shiny surface. Collodion of this consistency does not produce a membrane on the paraffin in drying, and therefore has no tendency to cause sections to roll. It has further the advantage that it penetrates to a certain depth below the surface of the preparation, and fixes the deeper layers of it in their places. The collodion must be diluted will ether as soon as it begins to show signs of leaving a shiny surface on the paraffin.

"Take the brush out of the collodion, wipe it against the neck of the bottle, so as to have it merely moist with collodion, and quickly pass it over the free surface of the preparation. Care must be taken not to let the collodion touch the vertical surfaces of the paraffin, especially not the one which is turned towards the operator, as that will probably cause the section to become stuck to the edge or under surface of the knife. As soon as the collodion is dry, which ought to be in two or three seconds, cut the section, withdraw the knife, and pass the collodion brush over the newly-exposed surface of the paraffin. Whilst this last layer of collodion is drying, take up the section from the knife and place it with the collodionised surface downwards on a slide prepared with fixative of Schaellibaum. Then cut the second section, and repeat the manipulations just described in the same order. A skilful operator can cut ribbons of sections, collodionising each section."

HENKING (*Zeit. f. wiss. Mik.*, iii, 4, 1886, p. 478) objects to the above process that the ether of the collodion softens the paraffin, and proposes a solution of *paraffin* in absolute alcohol. The solution is made by scraping paraffin into absolute alcohol.

For extremely brittle objects, such as ova of Phalangida, the same author recommends a thin (light yellow) solution of *shellac* in absolute alcohol.

HEIDER (*Embryonalentw. v. Hydrophilus*, 1889, p. 12; cf. *Zeit. f. wiss. Mik.*, viii, 4, 1892, p. 509) employs a solution made by mixing a solution of gum mastic in ether, of a syrupy consistency, with an equal volume of collodion, and diluting the mixture with ether until quite thin and liquid.

RABL (*ibid.*, xi, 2, 1894, p. 170) employs superheated paraffin, kept at a temperature of about 100° C. on a water-bath. This plan has the advantage of efficiently filling up any cavities there may be in the objects, and also of preventing the sections from rolling.

140. Clearing and Mounting.—The sections having been obtained are generally mounted on a slide in serial order by one of the methods described in the chapter on "Serial Section Mounting." If the sections have rolled or become folded on cutting they must be unrolled and smoothed out before being fixed to the slide.

The most efficacious plan for unrolling sections is perhaps the combined treatment with fluid and heat (GASKELL, *Quart. Journ. Mic. Sci.*, xxxi, 1890, p. 382; M. DUVAL, *Journ. de l'Anat. et de la Physiol.*, 1891, p. 26; HENNEGUY, *ibid.*, 1891, p. 398; GULLAND, *Journ. of Anat. and Physiol.*, 1891, p. 56; and others). The rolled sections are either floated on to the surface of warm water or warm alcohol contained in a watch-glass or suitable dish, which causes them to flatten out, and are then transferred to a slide to be mounted in any desired manner. Or the slide has a layer of water spread over it, the sections are laid on the water, and the slide is heated (to about 45° to 50° C.) until the sections flatten out, which happens in a few instants. The method can be made available for fixing series of sections to the slide; the further details necessary for the successful accomplishment of this are given in §§ 195 and 199.

VAN WALSEM (*Zeit. f. wiss. Mik.*, xi, 2, 1894, p. 228) describes a plan according to which the sections are arranged on a strip of parchment-paper which is moistened and passed over a warmed cylinder revolving in water on the principle of a postage-stamp dampener (see abstract with illustration in *Journ. Roy. Mic. Soc.*, 1895, p. 121).

The sections having been duly smoothed by one of these processes, and duly fixed to the slide (unless it is desired to keep them loose) all that now remains is to get rid of the paraffin and mount or stain as the case may be. The following solvents of paraffin have been recommended for freeing sections from the paraffin with which they are infiltrated:—Turpentine, warm turpentine, a mixture of 4 parts of essence of turpentine with 1 of creasote, creasote, a mixture of turpentine and oil of cloves, benzin, toluol, xylol, thin solution of Canada balsam in xylol (only applicable to very thin sections), hot absolute alcohol, naphtha, or any other paraffin oil of low boiling-point. Any of these may be used, but xylol and toluol are probably in most respects the best.

Naphtha does very well for sections that are to be passed through alcohol for staining, but is not safe for those that are to be put direct into balsam, as it frequently precipitates Canada balsam (with colophonium there is less risk).

If the slide be warmed to the melting-point of the paraffin, a few seconds will suffice to remove the paraffin if the slide be plunged into a tube of xylol or toluol. The sections may be mounted direct from the xylol or the slide may be brought into a tube of alcohol to remove the solvent for staining.

The removal of the paraffin is greatly facilitated by the following simple expedient (shown me by Professor v. Korotneff). Warm the slide over a flame, and whilst the paraffin is still melted hold it close before your lips and blow down on it vigorously. The paraffin and collodion or albumen or other medium used for gumming the sections to the slide are scattered right and left over the slide, leaving the sections high and dry. The splashes of paraffin are wiped off whilst warm with a cloth, and the slide being put at once into a tube of xylol, the remainder of the paraffin is dissolved out in a few seconds. This process also favours the adhesion of the sections.

141. **Recapitulation of the Paraffin Method**, as recommended to be practised.—Put into a small test-tube enough oil of cedar to cover your object. On to the oil pour carefully the same quantity of absolute alcohol. Take your (already dehydrated) object and put it carefully into the alcohol. Leave it until it has sunk to the bottom of the cedar oil. Wait till the refraction lines, § 111, have vanished. Then put it into paraffin kept at melting-point in a watch-glass. Let the paraffin be of the *very lowest melting point* that will give sufficiently thin sections, and to this end work in a cool place, so as not to be obliged to go above 45° C. if possible (see also next section). After a time change the paraffin (*i. e.* put the object into a fresh watch-glass with clean paraffin) once, or twice if the object be at all large. As soon as the object is thoroughly soaked with paraffin float the watch-glass on cold water. When cool, cut out a block of paraffin containing the object, and fix it with a heated needle on a cone of paraffin already mounted on the object-carrier of the microtome. Pare it square, and as close down to the object as possible on all sides except the one turned towards the knife; this had better have a wall of a millimètre or two, or more, according to the size of the object, left standing. Set the knife square. Set the block square to the knife-edge. Cut sections in chains or ribbons, collodionising them if necessary. Fix

them in serial order on a slide according to one of the methods given in Chap. XI. Warm, and remove the paraffin with xylol. Stain or mount.

Paraffin Masses.

142. Pure Paraffin.—It is now pretty generally admitted that pure paraffin forms an imbedding mass greatly superior for ordinary work to any of the many fatty mixtures that used to be recommended. I have only to repeat here that *a paraffin melting at* 45° C. is that which in my experience gives the best results *so long as the temperature of the laboratory is between* 15° *and* 17° C.; whilst for a temperature of 22° C. a paraffin melting at 48° is required. If the temperature of your laboratory have risen much above 22° C. you had better give it up, for good section-cutting with paraffin under such conditions is next to impossible.

Paraffin of the melting-points named is easily found in commerce. Intermediate sorts may be made by mixing hard and soft paraffin. Two parts of paraffin melting at 50° with one of paraffin melting at 36° C. give a mass melting at 48° C.

Many workers of undoubted competence prefer masses somewhat harder than those recommended, viz. of melting-points varying between 50° and 55° C. for the normal temperature of the laboratory. Some authors still recommend masses melting at 60° C. or higher. I can only repeat that I am convinced that, *besides being most hurtful to tissues, such masses have no* raison d'être *whatever in temperate climates.*

The figures above given have been repeatedly verified and are undoubtedly correct. But an important explanation remains to be made. The statements refer to work with the Thoma sliding microtome. I have since ascertained that microtomes with fixed knives, such as the Cambridge, the Minot, or the Reinhold-Giltay, will give good results, so far as cutting is concerned, with much harder paraffins. This is an advantage, so far as the obtaining of very thin sections is concerned; but it remains true that for delicate work it is well in the interest of the preservation of the tissues, to use a paraffin of as low a melting-point as possible.

Paraffin had better be obtained from Grübler, or one of the known dealers in microscopic reagents. Gaule recommends that the bluish transparent sorts be taken. I should say, transparent by all means, but, if possible, rosy rather than bluish. New paraffin is bluish; if kept long, which is well, it generally becomes rosy.

143. Prepared Paraffin (Pure).—GRAF SPEE (*Zeit. f. wiss. Mik.*, ii, 1885, p. 8) recommends the following preparation of commercial paraffin as giving a mass particularly favourable for ribbon-section cutting. Paraffin of about 50° C. melting-point is taken and heated in a porcelain capsule by means of a spirit lamp. After a time disagreeable white vapours are given off, and the mass shrinks a little. This result is arrived at in from one to six hours, according to the quality of the paraffin. The mass then becomes brownish yellow, and after cooling shows an unctuous or soapy surface on being cut. The melting-point will be found to have risen several degrees. This mass may be obtained ready prepared from Grübler.

BRASS (*loc. cit.*, p. 300) recommends the use of paraffin that has been kept for some years, as such has less tendency to crystallise than new paraffin. In this I concur.

FOETTINGER recommends (*Arch. de Biol.*, vi, 1885, p. 124) a somewhat complicated treatment with caustic potash, in which I have no faith (it was tried by one of the writers of the *Traité des Meth. techn.*, during the preparation of that work).

144. Paraffin Mixtures and other Similar Masses.—Of these the only ones that I think can be recommended for a moment are the following:—SCHULGIN'S paraffin with a little cerisin (this is evidently what Schulgin means by "ceresin"). Or, instead of cerisin, white wax (see *Zool. Anz.*, 1883, p. 21), or the mixture of BRASS (*Zeit. f. wiss. Mik.*, ii, 1885, p. 301), who recommends four to six parts of white wax to 100 of paraffin. Sections may be cleared with benzin. VAN WALSEM (*ibid.*, xi, 2, 1894, p. 216) advises for large objects 5 per cent. of *yellow* wax.

Soap Masses.

145. Utility of Soap Masses.—Soap masses certainly have many good points. The solvent is alcohol; the mass is highly *transparent*, very *penetrating*, and a good mass cuts far better that even paraffin. The mass may be cut either dry or with alcohol. As to the preservation of tissues, the mass is alkaline, which is against it; yet some workers still prefer soap to paraffin, and it has been recommended by so experienced a worker as Chun, for Siphonophora (certainly as delicate a class of objects as any that exist), on the ground of its producing less shrinkage than paraffin. It is evident that soap masses may render service for imbedding such structures as will not stand *complete* dehydration without shrinking. Soap imbedding is, in short, *a semi-wet* process.

146. Transparent Soap (PÖLZAM, *Morph. Jahrb.*, iii, 1877, 3tes Heft, p. 558).—The following account is taken from Salensky's paper on the gemmation of Salpa (*loc. cit.*).

Take good white soap ("gewöhnliche Kernseife"), cut it up into thin slices, and put them to dry in the sun for some days until they become white. The slices are then to be rubbed up to a fine powder, which is mixed with spirit to the consistency of porridge. Now mix the porridge with alcohol and glycerin in such proportions that the whole shall contain, for every ten parts by weight of the soap, 22 parts of glycerin and 35 parts of alcohol (90 per cent.). Let the whole simmer until there is obtained a perfectly transparent, syrupy, somewhat yellow fluid.

The objects, previously dehydrated in alcohol, are imbedded in this mass in the usual manner.

The mass may be removed from the sections either by means of water or of very dilute alcohol.

147. Transparent Soap (KADYI, *Zool. Anz.*, 37, 1879, vol. ii, p. 477).—Twenty-five grms. of shavings of stearate of soda soap (any stearate of soda soap will do, but the most to be recommended is the sort known in commerce as "weisse Wachskernseife") are heated in a retort with 100 c.c. of 96 per cent. alcohol over a water-bath until the whole is dissolved. Filter if necessary. If a drop of the solution be now poured into a watch-glass it will be seen that it almost immediately solidifies into a *white* mass. This is not what is wanted, and is a sign that the solution does not contain water enough.

Small quantities of water are therefore added by degrees to the solution, and the effect tested from time to time by pouring a drop of the mixture into a watch-glass. The mass will be seen to become more and more pellucid until a point is reached at which it is almost perfectly transparent, with merely the slightest blue opalescence. The preparation of the mass is then complete.

It is not possible to state *a priori* the exact proportion of water that should be added, as this naturally depends on the amount of water already present in the sample of soap taken. In very many cases it will be found that for about 120 grms. of soap solution 5 to 10 grms. of water will be required.

It is necessary to be very cautious in adding the water, as if too much be taken the mass solidifies more slowly or not at all. The greatest amount of elasticity and consistency is possessed by the mass at the moment in which it contains exactly the minimum amount of water necessary to make it transparent.

The reasons for this process are explained as follows:—Stearate of soda soap is soluble in divers proportions in warm alcohol. On cooling, the solution either solidifies into a homogeneous and pellucid mass, or into a white granular mass; or, in certain cases, does not solidify at all. The result in each case depends on the proportion of water present in the solution. For instance, if 5 to 6 parts of a tolerably dry soap be dissolved in 100 parts of 96 per cent. alcohol, a solution is generally obtained that solidifies into a transparent mass. But such a mass is too soft, and its melting-point too low; it melts by the heat of the finger. If now, in order to get a harder mass, you add more soap, you will get a solution that solidifies on cooling into a *white granular* mass; and it is only after adding to it a *certain* (*small*) quantity of water that you will obtain a solution that solidifies on cooling into a *transparent* mass. If you add more water than is just absolutely necessary to this end the mass will have too high a melting-point, and will solidify more slowly; and if still more water be added the solution will not solidify for hours, or, indeed, not at all. The more soap you have in your alcoholic solution the more water *must* you add in order to get a transparent mass, and the more *may* you add without depriving the solution of the faculty of solidifying. Besides the mass prepared in the proportions given above, useful masses may be made for certain purposes with 10, 20, 30, 40 per cent., or more or less of soap in alcohol. Weisker has employed a mass composed of about equal parts by weight of soap and alcohol. Such a mass is transparent, but yellow and oily, and takes a long time to solidify When cool it is very tough. It requires a considerable temperature to liquefy it, and has less penetrating power than the more alcoholic masses. It is, however, very suitable for hard, and especially for chitinous structures.

The mass recommended above boils at about 60° to 70° C. Objects should be imbedded in it in a watch-glass or in paper cases in the usual way. Whilst cutting, wet the knife and the mass with strong alcohol (one advantage of this method is that the knife remains perfectly clean). The sections are brought into 96 per cent. alcohol, which frees them from the mass instantaneously if warmed, and after a time if left cold.

REESE LIBRARY OF THE UNIVERSITY OF CALIFORNIA

Gelatin Masses.

148. Gelatin Imbedding is a method that has the advantage of being applicable to tissues that have not been in the least degree dehydrated, and may render great service in the study of *very watery objects*.

The *modus operandi* is, on the whole, the same as for other fusion masses, with the difference that the objects are prepared by penetration with *water* instead of alcohol or a clearing agent. After the cooling of the mass it may sometimes be cut at once, but it is generally necessary to harden it. This may be done by treatment for a few minutes with absolute alcohol (Kaiser), or for a few days with 90 per cent. alcohol (Klebs) or chromic acid (Klebs), or it may be frozen (Sollas).

The mass is removed from the sections by means of warm water.

149. Klebs' Gelatin (Glycerin Jelly) (*Arch. f. mik. Anat.*, v, 1869, p. 165).—A concentrated solution of isinglass is mixed with half its volume of glycerin.

150. Kaiser's Gelatin (*Bot. Centralb.*, i, 1880, p. 25; *Journ. Roy. Mic. Soc.*, iii, 1880, p. 504).—One part by weight of the finest French gelatin is left for about two hours in 6 parts by weight of water; 7 parts of glycerin are added, and for every 100 grms. of the mixture 1 grm. of concentrated carbolic acid. The whole is warmed for ten to fifteen minutes, stirring all the while, until the whole of the flakes produced by the carbolic acid have disappeared. Filter whilst warm through the finest spun glass, which has been previously washed in water and laid whilst wet in the filter.

151. Gerlach's Gelatin (Gerlach, *Unters. a. d. Anat. Inst. Erlangen,* 1884; *Journ. Roy. Mic. Soc.,* 1885, p. 541).—Take gelatin, 40 grms.; saturated solution of arsenious acid, 200 c.c.; glycerin, 120 c.c. Clarify with white of egg. The mass may be kept for years in a well-stoppered bottle. The objects to be prepared for imbedding by a bath of one-third glycerin.

152. Brunotti's **Cold Gelatin Mass** (*Journ. de. Botan.,* vi, 1892, p. 194; *Journ. Roy. Mic. Soc.,* 1892, p. 706).—Twenty grms. gelatin dissolved with heat in 200 c.c. distilled water, and 30 to 40 c.c. of glacial acetic acid with 1 grm. corrosive sublimate added after filtering. At the temperature of 15° C. the mass has the consistence of a thick syrup. Objects are prepared by soaking in some of the mass diluted with two to three vols. of water, then imbedded in the undiluted mass. The mass is then hardened in spirit or bichromate of potash, picric acid, or the like. No heat at all is required in this process.

CHAPTER X.

COLLODION (CELLOIDIN) AND OTHER IMBEDDING METHODS.

153. Advantages of the Collodion or Celloidin Method.—Collodion (or celloidin) masses do not require the employment of heat, which may be an important question in the case of some very delicate structures. They do not require that the objects should be cleared before imbedding, and that is an advantage in the case of very large objects. They are quite transparent, a quality which facilitates very greatly the orientation of the object. And they are specially indicated for very large objects, for the soaking in collodion being quite inoffensive to the most delicate elements may be prolonged if necessary for weeks, thus ensuring the harmless penetration of objects that would be literally cooked if they were submitted to a paraffin bath of like duration. Lastly, the mass being quite transparent, it is not necessary to remove it from the sections before staining and mounting them; it may remain, and fulfil the function of an admirable support to the tissues, holding in their places brittle or detached elements that without that help would fall to pieces and be lost.

There are two disadvantages. One is that the process is a very long one; as usually practised, the collodion process requires some three days for the imbedding of an object that can be imbedded in paraffin in an hour (though the time may be greatly abridged by GILSON's rapid process given below). Another is that it is impossible to obtain with celloidin sections as thin as those furnished by paraffin; the lowest limit I have been able to attain to is 7 μ, which for much work is not sufficient.

These considerations seem to justify the assertion that the collodion method is a special method, and not a general method.

As to the choice of a process, I urgently recommend the recently introduced practice of *clearing before cutting*, and *cutting dry* as described in §§ 166 and 168.

154. Collodion, Celloidin, and Photoxylin.—The collodion method is due to DUVAL (*Journ. de l'Anat.*, 1879, p. 185).

Celloidin, recommended later on by MERKEL and SCHIEFFERDECKER (*Arch. f. Anat. u. Phys.*, 1882, p. 200), is merely a patent collodion. It may be obtained through the post by writing to SCHERING'S "Grüne Apotheke," Wittick and Benkendorf, Berlin, N. Chaussée-Strasse, No. 19, or from GRÜBLER, or the other dealers in histological reagents. It is sent out in the form of tablets of a tough gelatinous consistency and slightly milky-white transparency. These tablets may, if desired, be dissolved at once in ether, or a mixture of ether and alcohol, to make a collodion of any desired strength. But it is better, as recommended by APÁTHY, to cut them up into thin shavings, which should be allowed to dry in the air until they become yellow, transparent, and of a horny consistency, and that these be then dissolved in alcohol and ether (sulphuric, free from acid). The solutions thus prepared are *free from the excess of water* that is present in the undried celloidin, and give after hardening a mass that is *more transparent* and of a better consistency for cutting (*Zeit. f. wiss. Mik.*, vi, 2, 1889, p. 164).

Imbedding masses of excellent quality can be prepared with ordinary collodion, but celloidin will be found more convenient to manipulate and furnishes more readily solutions of known concentration. Otherwise there is but little to choose between the two, and therefore in this work the terms collodion and celloidin are used indifferently.

Photoxylin (KRYSINSKY, VIRCHOW'S *Archiv*, cviii, 1887, p. 217; BUSSE, *Zeit. f. wiss. Mik.*, ix, 1, 1892, p. 47) is a dry substance, of the aspect of cotton wool, and chemically nearly related to celloidin. It can be obtained either from SCHERING or GRÜBLER. It gives a clear solution in a mixture of equal parts of ether and absolute alcohol, and should be used in exactly the same way as celloidin. In has the very great advantage of affording a mass which after hardening in 85 per cent. alcohol remains perfectly *transparent.* But celloidin or common collodion also give perfectly transparent masses *if cleared in bulk* as I recommend should be done (§§ 166—168); so that there is no advantage on this head in having recourse to photoxylin, unless it be desired to proceed in the old way.

155. Preparation of Objects.—The objects must first be thoroughly dehydrated with absolute alcohol. They are then soaked till thoroughly penetrated in ether, or, which is better, in a mixture of ether and absolute alcohol. DUVAL (*l. c.*) takes for this purpose a mixture of ten parts of ether to one of alcohol; SCHIEFFERDECKER (and the majority of workers) a mixture of equal parts of ether and alcohol; TUBBY (in *Nature*, November 17th, 1892, p. 51) advises a mixture of four parts of ether and one of alcohol. But the point is one of no great importance.

This stage may be omitted if the objects are of a sufficiently permeable nature, and they may be brought direct from alcohol into the collodion bath.

156. The Collodion Bath.—The next step is to get the objects penetrated with thick collodion. The secret of success here is to penetrate them first with thin solutions, then with the definitive thick one. (A thin solution may be taken to mean one containing from 4 to 6 per cent. of celloidin (dried as described in § 154); a thick solution one containing 10 to 12 per cent.)

If collodion be taken, the thin solution may be made by diluting it with ether. If photoxylin or celloidin be taken, the solutions are made in a mixture of ether and absolute alcohol in equal parts.

The dried celloidin shavings dissolve very slowly in the mixture. ELSCHNIG (*Zeit. f. wiss. Mik.*, x, 4, 1893, p. 443) states that solution is obtained much quicker if the shavings be first allowed to swell up for twenty-four hours in the necessary quantity of absolute alcohol, and the ether be added afterwards. On trial it seems to me that this is so.

BUSSE (op. cit., ix, 1, 1892, p. 47) gives the following proportions for the successive baths:—No. 1, 10 parts by weight of photoxylin or perfectly dried celloidin to 150 parts of the ether and alcohol mixture: No. 2, 10 parts of photoxylin or celloidin to 105 of the mixture; No. 3, 10 parts to 80 of the mixture (already used solution may be employed for the first bath).

I generally use only two solutions: one weak one, and one strong one corresponding approximately to Busse's No. 2. His No. 3 is so thick that excessive time is required to obtain penetration by it.

The objects ought to remain in the first bath until very thoroughly penetrated ;—days, even for small objects,—weeks or months for large ones (human embryos of from six to twelve weeks, for instance). If the object contain cavities, these should be opened to ensure their being filled by the mass.

When the object is duly penetrated by the thin solution, or solutions if more than one have been employed, it should be brought into the thickest one. This may be done (as first described in this work, 1st. edit., 1885, p. 194) by allowing the thin solution to concentrate slowly (the stopper of the containing vessel being raised, for instance by means of a piece of paper placed under it), and making up the loss from evaporation with thick solution.

157. Imbedding.—*If the object is such that it can be fixed*, by gumming or otherwise, *to the holder of the microtome without the intervention of any specially shaped mass of collodion around it*, and if the presence of such a mass be not required in the interest of the orientation of the object or of the production of continuous series of sections, *no special imbedding is necessary*, and as soon as the objects are duly penetrated by the thick solution you may proceed to the hardening part of the process. In other words, it is waste of time to get the object into a special block of collodion if that is not rendered desirable for the reasons above mentioned. If, however, it be desirable, the objects must at this stage, if it has not been done before, be *imbedded*—that is, arranged in position in the thick collodion in the receptacle in which they are to be hardened. For the usual manipulations see § 129. I recommend the paper thimbles or cylindrical trays, Fig. 2, as being very convenient for collodion imbedding. The bottoms, however, should be made of soft wood in preference to cork ; cork is elastic, and bends in the object-holder of the microtome, deforming the mass and object. The box should be prepared for the reception of the object by pouring into it a drop of collodion, which is allowed to dry. The object of this is to prevent bubbles coming up through the wood or cork and lodging in the mass.

Objects may also be imbedded on a piece of pith or leather, which should also be prepared with a layer of dry collodion.

Watch-glasses, square porcelain water-colour moulds, and the like, also make convenient imbedding receptacles. Care should be taken to have them perfectly *dry*.

It not unfrequently happens that during these manipulations bubbles make their appearance in the mass. Before proceeding with the hardening these should be got rid of. This may be done by exposing the whole for an hour or two to the vapour of ether in a desiccator or other well-closed vessel. Care should be taken that the ether (which may be poured on the bottom of the vessel) does not wet the mass (BUSSE, *Zeit. f. wiss. Mik.*, viii, 4, 1892, p. 467).

158. Orientation.—If it be desired to mark the position of the object in the mass in order to facilitate the subsequent orientation of it on the object-holder of the microtome, recourse may be had to the method described by EYCLESHEIMER in *Amer. Nat.*, xxvi, 1892, p. 354 (see also *Journ. Roy. Mic. Soc.*, 1892, p. 562). The object is imbedded in one of the metal boxes described in § 129. The box has its ends and sides perforated at regular intervals by small opposite holes. Silk threads are passed through these holes from side to side, stretched, and kept tight by sticking them to the sides of the box by means of a drop of celloidin, leaving a length of a couple of inches hanging loose. The loose ends are soaked in thin celloidin solution with which lamp-black has been mixed. The object is arranged in position on the framework formed by the taut threads in the box, the mass is poured in, and the whole is hardened. After hardening, the celloidin holding the ends of the threads is dissolved by means of a drop of ether, and the lampblacked ends are pulled through the box. This leaves adhering to the bottom of the mass a series of black lines which form orientation points.

APÁTHY (*Zeit. f. wiss. Mik.*, v, 1, 1888, p. 47) arranges objects on a small rectangular plate of gelatin, placed on the bottom of the imbedding-recipient. The gelatin is turned out with the mass after hardening, and cut with it. The edges of the gelatin form good orientation lines.

HALLE and BORN (see *Zeit. f. wiss. Mik.*, xii, 3, 1896, p. 364) use plates of hardened white of egg, in which a shallow furrow for the reception of the objects has been cut by means of a special instrument.

159. Hardening, Preliminary.—This is logically the next step, but as a matter of fact is frequently begun before. For the different processes of the collodion method so run into one another that it is difficult to assign natural lines of demarcation between them.

The objects being imbedded, and in the stage at which we left them at the end of § 156, the treatment should be as follows. The receptacles or supports are set with the mass under a glass shade, allowing of just enough communication with the air to set up a slow evaporation. Or porcelain moulds or small dishes may be covered with a lightly fitting cover.

As soon as the added thick collodion (of which only just enough to cover the object should have been taken) has so far sunk down that the object begins to lie dry, fresh thick solution is added, and the whole is left as before. (If the first layer of collodion has become too dry, it should be moistened with a drop of ether before adding the fresh collodion.) Provision should be again made for slow evaporation, either in one of the ways above indicated, or, which is perhaps better, by setting the objects under an *hermetically* fitting bell-jar, which is lifted for a few seconds only once or twice a day. (I have sometimes found it advantageous to set the object under a bell-jar together with a dish containing alcohol, so that the evaporation is gone through in an atmosphere of alcohol. This is especially indicated for very large objects.) The whole process is repeated every few hours for, if need be, two or three days.

When the mass has attained a consistency such that the ball of a finger (*not* the nail) no longer leaves an impress on it, it should be scooped out of the dish or mould, or have the paper removed if it has been imbedded in paper, and be submitted to the next stage of the hardening process. (If the mass is found to be not quite hard enough to come away safely it should be put for a day or two into weak alcohol, 30 to 70 per cent.)

160. Hardening, Definitive.—Several methods are available for the definitive hardening process. One of these is the *chloroform* method, due to VIALLANES (*Rech. sur l'Hist. et le Dév. des Insectes*, 1883, p. 129). I recommend this method for *small* objects, because I find it much more rapid than the alcohol method, whilst giving at least as good a consistency to the mass. (SCHIEFFERDECKER does not find this, v. *Zeit. f. wiss. Mik.*, v, 4, 1881, p. 506.) For *large* objects the method is said to be inferior to the alcohol method, because the rapid hardening of the external layers is an obstacle to the diffusion necessary to the hardening of the inner layers.

The method consists in bringing the objects into *chloroform*. "Under the influence of this reagent this collodion coagulates into a mass having the consistence of wax, but having also an elasticity that renders it unbreakable, and having besides the precious quality of being admirably transparent, and possessing exactly the index of refraction of glass."

In some cases a few *hours'* immersion is sufficient to give the requisite consistence. In no case have my specimens required more than three days. But the length of time required varies in a very inexplicable way, so that no rule can be given. The collodion frequently becomes opaque on being put into the chloroform, but regains its transparency after a time.

Small objects may be hardened by chloroform *without preliminary hardening by evaporation.* All that is necessary is to expose the mass to the air for a few seconds until a membrane has formed on it, and then bring it into chloroform. If the mass is in a test-tube this may be filled up with chloroform, and left for two or three days. By this time the collodion mass will be considerably hardened, and also somewhat shrunk, so that it can be shaken out of the tube. It is then brought into fresh chloroform in a larger vessel, where it remains for about six days, after which time it is generally ready for cutting. The process is sometimes much more rapid than this.

Good chloroform is a necessity, as the reaction cannot be obtained with samples of chloroform that are not free from water.

The above processes are excellent, but I regard them as primitive forms of the chloroform method. I now almost always harden in *vapour of chloroform.* All that is necessary is to put the liquid mass (after having removed bubbles as directed in § 157) with its recipient into a desiccator on the bottom of which a few drops of chloroform have been poured. The action is very rapid, and the final consistency of the mass at least equal to that obtained by the best alcohol-hardening. We shall revert to this subject, § 168.

The more commonly employed hardening method is the *alcohol* method. The objects are thrown into alcohol and left there until they have attained the right consistency (one day to several weeks). The bottle or other vessel containing the alcohol *ought not to be tightly closed, but should be left at least partly open.*

The strength of the alcohol is a point on which the practice of different writers differs greatly. The question may now be considered to be finally settled by experiments, specially directed to the clearing up of this point, made by BUSSE (*Zeit. f. wiss. Mik.*, ix, 1, 1892, p. 49), and which I have repeated and confirmed. BUSSE finds that *alcohol of about* 85 *per cent.* is the best, *both as regards the cutting consistency and the transparency* of the mass. Care must be taken to keep the mass moist whilst cutting, as it dries by evaporation very quickly.

Lastly, the mass may be *frozen.* After preliminary hardening by alcohol it is soaked for a few hours in water in order to get rid of the greater part of the alcohol (the alcohol should not be removed entirely, or the mass may freeze too hard). It is then dipped for a few moments into gum mucilage in order to make it adhere to the freezing plate, and is frozen. The sections are brought into warm water. If the mass have frozen too hard, cut with a knife warmed with warm water.

A paper has been written by FLORMAN (*Zeit. f. wiss. Mik.*, vi, 2, 1889, p. 184) to recommend that the definite hardening should be done without the aid of alcohol or chloroform, by simply cutting out the blocks, turning them over, and carefully continuing the evaporation process in the way described above. I described this process myself in the first edition of this work. No doubt the author is right in claiming for it a superior degree of hardening of the mass; but I doubt whether it is possible to carry the hardening much beyond the point attained by the chloroform or alcohol method without incurring a very undesirable degree of shrinkage.

The hardening processes used in the method of clearing before cutting, which I prefer to all the foregoing, will be described later on, § 168.

161. Preservation.—The hardened blocks of collodion may be preserved till wanted in weak alcohol (70 per cent.). They may also be preserved dry by dipping them into melted paraffin (APÁTHY, *Zeit. f. wiss. Mik.*, v, 1, 1888, p. 45).

Reference numbers may be written with a soft lead pencil on the bottom of the paper trays, or with a yellow oil pencil on the bottom of the watch-glasses in which the objects are imbedded. On removal of the paper from the collodion after hardening, the numbers will be found impressed, on the collodion.

162. Cutting.—If the object has not been stained before imbedding, it will form so transparent a mass with the collodion that the arrangement of the object and sections in the right position may be rendered very difficult. It is, therefore, well to stain the collodion lightly, just enough to make its outlines visible in the sections. This may be done by adding picric acid or other suitable colouring matter dissolved in alcohol to the collodion used for imbedding, or to the bergamot oil used for clearing.

To fix to the microtome, proceed as follows. Take a piece of soft wood, or, for very small objects, pith, of a size and shape adapted to fit the holder of the microtome. Cover it with a layer of collodion, which you allow to dry. Take the block of collodion, or the impregnated and hardened but not imbedded object, cut a slice off the bottom, so as to get a clean surface; wet this surface first with absolute alcohol, then with ether (or allow it to dry), place one drop of *very thick* collodion on the prepared wood or pith, and press down *tightly* on to it the wetted or dried surface of the block of collodion. Then throw the whole into weak (70 per cent.) alcohol for a few hours (or even less), or into chloroform, or vapour of chloroform, for a few minutes, in order that the joint may harden.

Dr. Lindsay Johnson informs me that he finds it very convenient to take for this purpose the cement used by metal turners for fastening metal objects on to boxwood chucks. The exact composition of this cement varies somewhat, but an average one is—beeswax, 1 part; resin, 2 parts. To use it, you must get the block of celloidin perfectly dry at the bottom, then warm the object-holder slightly, if possible, over a flame; drop on to it a few drops of melted cement, and press on to it the block of celloidin, which will be firmly fixed as soon as the cement is cool—that is, in a few seconds.

For objects of any considerable size it is important not to use cork for mounting on the microtome, especially if the object-holder be a vice; for cork bends under the pressure of the holder, and the elastic collodion bends with it, deforming the object. I have seen large embryos so deformed in this way that the sections obtained were true calottes, segments of a sphere. If the object-holder be of the cylinder type, as in the later forms of the Thoma microtome, the above-described accidents will be less likely to happen, and a good cork may be used; but even then, I think, wood is safer. GAGE has recommended bits of glass cylinders. JELINEK (*Zeit. f. wiss. Mik.*, xi, 2, 1894, p. 237) recommends a sort of vulcanite known as "Stabilit," which is manufactured for electrical insulation purposes. It is supplied in suitable blocks by HERMANN DÜMLER, 4, Schwarzpanierstrasse, Vienna, ix, 3 (presumably also obtainable through GRÜBLER AND CO.).

Sections are cut with a knife kept abundantly wetted with alcohol (of 50 to 85 or even 95 per cent.). Some kind of drip arrangement will be found very useful here. Apáthy recommends that the knife be smeared with yellow vaselin; it cuts better, is protected from the alcohol, and the mobility of the alcohol on the blade is lessened.

The knife is set in as oblique a position as possible.

Very brittle sections may be collodionised as explained above (§ 139).

The sections are either brought into alcohol (of 50 to 85 or 95 per cent.) as fast as they are made; or if it be desired to mount them in series, they are treated according to one of the methods described below, in the chapter on "Serial Section Mounting."

163. Staining.—The sections may now be stained as desired, either loose, or mounted in series on slides or on paper as described in the chapter on "Serial Section Mounting." It is *not* in general necessary, nor indeed desirable, to remove the mass before staining, as it usually either remains colourless or gives up the stain on treatment with alcohol. But some of the anilin dyes and some other colours stain it strongly, and are not removed with sufficient completeness by the processes of dehydration and clearing. If it be desired to employ these, the mass may be removed by treating the sections with absolute alcohol or ether.

164. Clearing and Mounting.—You may mount in glycerin without removing the mass, which remains as clear as glass in that medium.

You may mount in balsam, also without removing the mass, which does no harm, and serves the useful purpose of holding the parts of the sections together during the manipulations. Dehydrate in alcohol of 95 or 96 per cent. (not absolute, as this attacks the collodion). NIKIFOROW (*Zeit. f. wiss. Mik.*, viii, 2, 1891, p. 189) recommends a mixture of equal parts of alcohol and chloroform. Clear with a substance that does not dissolve collodion. The clearing agents most recommended are origanum oil (*Ol. Origan. Cretici,* it is said, should be taken, not *Ol. Orig. Gallici;* but see, as to this reagent, the remarks in Chap. VII, § 118), bergamot oil (said to make sections shrink somewhat), oil of sandal-wood, lavender oil, oil of cedar-wood (safe and gives excellent results, but acts rather slowly), chloroform, xylol or benzin (may make sections shrink if not well dehydrated), or Dunham's mixture of three or four parts of white oil of thyme with one part of oil of cloves. (As to oil of thyme, see also "Origanum Oil" in Chap. VII, § 118).

FISH (*Proc. Amer. Mic. Soc.,* 1893) advises a mixture of one part of red oil of thyme with three parts of castor oil, the latter being added in order to counteract the volatility of the thyme oil. But later (June, 1895), writing to me, Dr. Fish say he has substituted the white oil of thyme for the red, and finds it an advantage in orientating. See also § 119.

Some specimens of clove oil dissolve collodion very slowly, and may be used, but I would not be understood to recommend it. The action of origanum oil varies much, acccording to the samples; some sorts do not clear the collodion, others dissolve it, others pucker it. MINOT (*Zeit. f. wiss. Mik.,* iii, 2, 1886, p. 175) says that Dunham's mixture "clarifies the sections very readily and softens the celloidin just enough to prevent the puckering, which is so annoying with thyme alone."

Carbolic acid has been recommended. WEIGERT (*Zeit. f. wiss. Mik.,* iii, 4, 1886, p. 480) finds that a mixture of 3 parts of xylol with 1 part of carbolic acid (anhydrous) clears well. But it must not be used with the basic anilin stains, as it discolours them. For these, anilin oil may be used with the xylol, in the place of carbolic acid.

Anilin oil clears well (it will clear from 70 per cent. alcohol), but unless thoroughly removed the preparation becomes yellowish brown. It may be removed by soaking in chloroform for twenty-four hours (see VAN GIESON, *Amer. Mon. Mic. Journ.,* 1887, p. 49, or *Journ. Roy. Mic. Soc.,* 1887, p. 519, for a review of these clearing agents; see also § 125).

Beechwood creasote has been recommended (by M. Flesch).

EYCLESHEIMER (*Amer. Nat.,* xxvi, 1892, p. 354; *Journ. Roy. Mic. Soc.,* 1892, p. 565) advises a mixture of equal parts of bergamot oil, cedar oil, and carbolic acid.

165. Review of the Older Celloidin Method.—The older celloidin method, described in the foregoing pages, is extremely lengthy and cumbrous. The operation of infiltrating the tissues with the collodion requires days or weeks. The hardening process requires nearly as much time. The resulting mass has the disadvantage of being opaque or at most only translucent, not transparent. The mass has to be cut under the surface of alcohol, or at least with constant wetting with alcohol and with a knife kept constantly wet with alcohol. By the recent method of clearing the mass before cutting, a large part of these defects is done away with; the resultant mass is as clear as glass, thus allow- the most perfect orientation of the object; and as I have shown (LEE et HENNEGUY, *Traité des Méthodes techniques de l'Anat. Mic.*, 1896, p. 230) the mass can with advantage be cut *dry*, thereby greatly simplifying the operation of cutting. By GILSON's ingenious *Rapid Method*, the time necessary for hardening is very greatly abridged, and the whole series of operations becomes almost as short and simple as the paraffin method. I cannot imagine that anyone who has ever employed the new method would willingly go back to the old one. The following paragraphs describe the new method.

166. The New Method, by Clearing before Cutting.—This process is due, I believe, in the first instance to BUMPUS (*Amer. Natur.*, xxvi, 1892, p. 80; see *Journ. Roy. Mic. Soc.*, 1892, p. 438). He advises clearing the mass, after hardening in chloroform, with white oil of thyme or other suitable clearing agent (see above, § 164). After clearing, the under surface of the block of mass is washed with ether, and cemented with thick celloidin solution to a block of wood for cutting, in the manner described above, the whole being thrown into chloroform for a few minutes to harden the joint. The knife is wetted with the clearing oil, and the same oil is employed for covering the exposed surface of the object after each cut. Similar recommendations are made by EYCLESHEIMER (op. cit., pp. 354, 563), carbolic acid, or glycerin, or the mixture given § 164, being suggested for clearing. Professor GILSON writes me that he has for some time past adopted the practice of clearing before cutting with cedar oil, as described below, § 167.

FISH (*loc. cit.*, § 164) also advocates the practice of clearing in the mass, recommending the clearing mixture there given.

All the authors above quoted cut in the *wet* way, that is to say, with a knife wetted with the clearing liquid. I have found a great improvement in cutting *dry*, and in employing the combined hardening and clearing process of GILSON, given below.

167. GILSON'S **Rapid Process.**—The following *rapid method* communicated to me by Prof. GILSON (April, 1892) has the advantage of being the most expeditious of any. The object is dehydrated, soaked in ether, and brought into a test-tube with collodion, or thin celloidin solution. The tube is dipped into a bath of melted paraffin, and the collodion allowed to boil (which it does at a very low temperature) until it has become of a syrupy consistence. It should be boiled down to about one third of its volume. The mass is then turned out, mounted on a block of hardened celloidin, and the whole hardened in chloroform or in a mixture of chloroform and cedar oil for about an hour. It is then cleared in cedar oil (if hardened in pure chloroform; special clearing will not be necessary if it has been hardened in the mixture). It may now be fixed in the microtome and cut, using cedar oil to wet the knife, and cover the exposed surface of the object after each cut.

It will be observed that this process is very much more rapid than the old process, in two ways—the celloidin bath, being given warm, is greatly abridged; small objects can be duly infiltrated in an hour, where days would be required by the old process. The hardening is also much more rapid than hardening by alcohol, which requires at least twenty-four hours. As collodion boils at a very low temperature, very little heat is required, and there is no risk of the tissues suffering on that head.

168. The Dry Cutting Method.—I recommend the following as being a further improvement. Penetrate with collodion or celloidin either by GILSON'S process, or by soaking in the cold in the usual way, § 156. This is a much slower process, but does not take up more of the worker's time, as the specimens require no attention whilst in the bath. Imbed as usual, either directly on the holder of the microtome, or in a paper tray or a water-colour mould or the like. Harden in vapour of chloroform for from one hour (generally sufficient for small objects) to overnight. This is done by putting the preparation into a Steinach's sieve-dish or into a desiccator, on the bottom of which a tea-spoonful of chloroform has been poured. (The objects may remain for months in the chloroform vapour if desired.) As soon as the mass has attained sufficient superficial hardness, it is, of course, well to turn it out of its recipient, and turn it over from time to time, in order that it may be equally exposed on all sides to the action of the vapour. When fairly hard (it is not necessary to wait till the mass has attained all the hardness of which it is susceptible), throw it into GILSON's mixture. This should be at first a mixture of one part of chloroform with one or two parts of cedar oil. From time to time more cedar oil should be added, so as to bring the mixture up gradually to nearly pure cedar oil. As soon as the object is cleared throughout, the mass may be exposed to the air, and the rest of the chloroform will evaporate gradually. The block may now be mounted on the holder of the microtome with a drop of thick collodion, § 162, and may either be cut at once, or may be preserved indefinitely without change in a stoppered bottle. *Cut dry*, the cut surface will not dry injuriously under several hours. The cutting quality of the mass is often improved by allowing it to evaporate in the air for some hours.

The hardening may be done at once in the chloroform and cedar-wood mixture, instead of the chloroform vapour, but I find the latter process preferable as giving a better hardening. And clearing may be done in pure cedar oil instead of the mixture, but then it will be very slow, whereas in the mixture it is extremely rapid.

169. Double Imbedding in Collodion and Paraffin.—This complicated process is sometimes, though rarely, indicated for objects of which it is desired to have very thin sections, and which are too brittle to give good sections by the plain paraffin process.

KULTSCHIZKY's **Method** (*Zeit. f. wiss. Mik.*, iv, 1, 1887, p. 48).—After the collodion bath, the object is soaked in oil of origanum (*Oleum Origani vulg.*). It is then brought into a mixture or origanum oil and paraffin, heated to not more than 40° C., and lastly into a bath of pure paraffin.

The mass may be preserved in the dry state, and may be cut dry.

RYDER (*Queen's Micr. Bull.*, 1887, p. 43; *Journ. Roy. Mic. Soc.*, 1888, p. 512) modified the process by substituting chloroform for the origanum oil.

UNIVERSITY

Ide (*La Cellule*, vii, 1891, p. 347, and viii, 1, 1892, p. 114) employed with success the following method :—The object is imbedded in collodion in a tube by Gilson's process (*supra*, § 167) ; the collodion is boiled for forty minutes, then brought for fifteen minutes (this is for small objects) into chloroform heated to 30° C. containing one fourth part of paraffin dissolved in it, then for ten minutes into pure melted paraffin.

Field and Martin (*Bull. Soc. Zool. de France*, 1894, p. 48), finding that it is difficult to get hardened celloidin masses adequately impregnated with the paraffin, have worked out the following process of *simultaneous* imbedding. A solution of dried celloidin in a mixture of equal parts of absolute alcohol and toluene, of about the consistency of clove oil, is made. This solution is saturated with paraffin, added in shavings at a temperature not exceeding 20° to 23° C. The tissues are prepared by soaking in some of the mixture of alcohol and toluene, and are then penetrated with the celloidin-paraffin solution. The mass is hardened by throwing it into a saturated solution of paraffin in chloroform or in toluene, and is finally imbedded in pure paraffin in the usual way.

Other Evaporation Masses.

170. Joliet's Gum and Glycerin Method (*Arch. Zool. exp. et gén.*, x, 1882, p. xliii ; *Journ. Roy. Mic. Soc.* [N.S.], ii, 1882, p. 890).—Pure gum arabic dissolved in water to the consistency of a thick syrup. (Solutions of gum sold under the name of strong white liquid glue [" *colle forte blanche liquide à froid* "] may also be used ; they have the advantage of having a uniform consistency.*) Pour a little of the solution into a watch-glass, so as not quite to fill it, add from 6 to 10 drops of pure glycerin, stir until thoroughly mixed.

Between the limits of 6 to 10 drops of glycerin the proportions most suitable to the nature of the object and to the season of the year must be found by experimental trials. In the winter or in rainy weather less glycerin should be taken than in the summer or dry weather.

It is often well to soak the object in glycerin before putting it into the mass. In this case less glycerin should be added to the gum, in proportion to the amount of glycerin contained in the object.

The object is imbedded in the mass in the watch-glass, and the whole left to dry for from one to four days. When it has assumed a cartilaginous consistency, a block containing the object is cut out, turned over, and allowed to dry again until wanted for use. A stove, or the sun, may be employed for drying, but it is best to dry slowly at the normal temperature. The block may be preserved in good condition almost indefinitely, the gum, when mixed with a sufficient quantity of glycerin, never becoming hard or brittle. It is generally better to wait till the blocks have assumed such a consistency that they cannot be easily bent. It is after having waited almost a week that the author always obtained the best sections. The gum is dissolved out from the sections by means of a drop of water on the slide. The sections are then covered, and a drop of glycerin being added, the preparation is complete as soon as the water has evaporated.

---

* It is highly probable that these commercial preparations contain gelatin, and perhaps some other gum besides gum arabic.

This process may render service occasionally in the study of extremely watery organisms, such as *Salpa*, or the Ctenophora.

171. **Stricker's Gum Method** (*Hdb. d. Gewebel.*, p. xxiv).—A concentrated solution of gum arabic. The object may be prepared in alcohol and imbedded in the gum in a paper case. The whole is thrown into alcohol, and after two or three days may be cut.

I have seen masses of admirable consistency prepared by this simple method.

172. ROBERTSON's **Grape-sugar Method,** see *Journ. of Anat. and Physiol.*, xxiv, 1890, p. 230; *Zeit. f. wiss. Mik.*, vii, 1, 1890, p. 33.

173. **Hyatt's Shellac Method,** see *Am. M. Micr. Journ.*, i, 1880, p. 8; *Journ. Roy. Mic. Soc.*, iii, 1880, p. 320.

This process is merely intended for the purpose of making sections through hard chitinous organs consisting of several pieces, such as stings and ovipositors, retaining all the parts in their natural positions.

174. **Von Koch's Copal Method** (*Zool. Anz.*, 2, vol. i, 1878, p. 36).—Small pieces of the object are stained in bulk and dehydrated with alcohol. A thin solution of copal in chloroform is prepared by triturating small fragments of copal in a mortar with fine sand, pouring on chloroform to the powder thus obtained, and filtering. The objects are brought into a capsule filled with the copal solution. The solution is now slowly evaporated by gently heating the capsule on a tile by means of a common night-light placed beneath it. As soon as the solution is so far concentrated as to draw out into threads that are brittle after cooling, the objects are removed from the capsule and placed to dry for a few days on the tile, in order that they may more quickly become hard. When

they have attained such a degree of hardness that they cannot be indented by a finger-nail, sections are cut from them by means of a fine saw. The sections are rubbed down even and smooth on one side with a hone, and cemented, with this side downwards to a slide, by means either of Canada balsam or copal solution. The slide is put away for a few days more on the warmed tile. As soon as the cement is perfectly hard the sections are rubbed down on a grindstone, and then on a hone, to the requisite thinness and polish, washed with water, and mounted in balsam.

The process may be varied by imbedding the objects unstained, removing the copal from the sections by soaking in chloroform, decalcifying them if necessary, and then staining.

It is sometimes a good plan after removing the copal, to cement a section to a slide by means of hard Canada balsam, then decalcify cautiously the exposed half of the specimen, wash, and stain it. In this way von Koch was able to demonstrate the most delicate lamellæ of connective tissue in *Isis elongata*.

This method was imagined in order to enable the hard and soft parts of corals to be studied in their natural relations. It is evidently applicable to the study of any structures in which hard and soft parts are intimately combined. For purposes such as these it is certainly a method of the greatest value.

175. Ehrenbaum's Colophonium and Wax Method (*Zeit. f. wiss. Mik.*, 1884, p. 414).—Ehrenbaum recommends that the objects be penetrated by a mass consisting of ten parts of colophonium to one of wax. The addition of wax makes the mass less brittle. Sections are obtained by grinding in the usual way. The mass is removed from them by means of turpentine followed by chloroform.

176. Weil's Canada Balsam Method (*Zeit. f. wiss. Mik.*, v, 2, 1888, p. 200).—Balsam heated till brittle when cold, then dissolved in chloroform. Heat the objects in the mass on a water-bath. For further details see *Journ. Roy. Mic. Soc.*, 1888, p. 1042.

Congelation Masses.

177. The Freezing Method.—Fresh tissues may be, and are, frequently frozen without being included in any mass, and in certain cases very satisfactory sections can be obtained in this manner. But the formation of ice crystals frequently causes tearing of delicate elements, and it is better to infiltrate the tissues with a mass that does not crystallise in the freezing mixture, but becomes hard and tough. Gum arabic affords such a mass. Some workers used common gum water, which is either poured into the well of the microtome or round the object on the object plate, according to the form of microtome used.

178. Syrup and Gum Congelation Mass (Hamilton, *Journ. of Anat. and Phys.*, xii, 1878, p. 254).—The hardening reagent having been soaked out by water, the tissues are prepared for freezing in the following manner, which it is important to observe, otherwise it will be found that the crystals of ice so break up delicate tissue as to render it totally useless for minute examination. The tissues are to be well soaked in syrup. The sugar somewhat retards the freezing, and besides, seems to alter the manner of crystallisation, so that instead of the ice being spicular in form it becomes granular, and does no injury to the parts.

The syrup requires to be of a particular strength, viz. double refined sugar, 2 ounces; water, 1 fluid ounce.

Wash the superfluous syrup from the surface, and put into the ordinary mucilage for an hour or so before cutting. Imbed in the freezing microtome with mucilage in the usual way. Float the sections into water.

179. Gum and Syrup Congelation Mass (Cole, *Methods of Microscopical Research*, 1884, p. xxxix; *Journ. Roy. Mic. Soc.* [N.S.], iv, 1884, p. 318).—Gum mucilage (B.P.), 5 parts; syrup, 3 parts. (For brain and spinal cord, retinæ, and all tissues liable to come in pieces put 4 parts of syrup to five of gum.) Add 5 grains of pure carbolic acid to each ounce of the medium.

(Gum mucilage [B.P.] is made by dissolving 4 ounces of picked gum acacia in 6 ounces of water.)

The syrup is made by dissolving 1 pound of loaf sugar in 1 pint of water and boiling.

This medium is employed for soaking tissues previous to freezing. They may remain in it for "any length of time, all the year round" if desired.

The freezing is conducted as follows:—the gum and syrup is removed from the *outside* of the object by means of a cloth; the spray is set going and a little gum mucilage painted on the freezing plate; the object is placed on this and surrounded with gum mucilage; it is thus saturated with gum and syrup, but surrounded when being frozen with mucilage only. This combination prevents the sections from curling up on the one hand, or splintering from being too hard frozen on the other. The mass ought to cut like cheese. Should freezing have been carried too far, wait for a few seconds.

180. Dextrin Congelation Mass (Webb, *The Microscope*, ix, 1890, p. 344; *Journ. Roy. Mic. Soc.*, 1890, p. 113).—Thick solution of dextrin in solution of carbolic acid in water (1 in 40). Use heat for making the solution if desired. This medium is much cheaper than the gum and syrup mass, and, according to Webb, possesses superior cutting qualities.

181. Gelatin Congelation Mass (Sollas, *Quart. Journ. Mic. Soc.*, xxiv, 1884, pp. 163, 164; *Journ. Roy. Mic. Soc.* [N.S.], iv, 1884, p. 316).—"Instead of gum one uses gelatin jelly. This is prepared and clarified in the usual manner. It should set into a stiff mass when cold. . . . . The tissue to be cut is transferred from water to the melted jelly, and should remain in it till well permeated."

The sections are transferred to a slide as soon as cut. On touching the glass they adhere to it. When enough sections have been thus arranged they are covered with a drop of glycerin; a cover is put on, and the mount closed with any suitable cement. In process of time the glycerin will permeate the gelatin and convert it into glycerin jelly; this may be hastened by placing the slide in an oven kept at about 20° to 30° C.

182. Gum-Gelatin Congelation Mass (Jacobs, *Amer. Natural.*, 1885, p. 734; *Journ. Roy. Mic. Soc.*, 1885, p. 900).—Gum arabic, 5 parts; gum tragacanth, 1 part; gelatin, 1 part. Dissolve in enough warm water (containing one sixth of glycerin) to give a mass of the consistency of thin jelly when cold.

183. White of Egg Congelation Mass (ROLLETT, *Denkschr. math. naturw. Kl. k. Acad. Wiss. Wien*, 1885; *Zeit. f. wiss. Mik.*, 1886, p. 92).—Small portions of tissue brought in the white of a freshly laid egg on to the freezing stage, frozen, and cut. The knife must be well cooled.

184. Oil of Aniseed Congelation Mass (KÜHNE, *Centralb. f. Bakteriol.*, xii, 1892, p. 28; *Journ. Roy. Mic. Soc.*, 1892, p. 706).—Soak in oil of aniseed for twelve to twenty-four hours, freeze and cut, and remove the oil from the sections by means of alcohol.

V. A. MOORE (*Amer. Mon. Mic. Journ.*, 1894, p. 373; *Journ. Roy. Mic. Soc.*, 1895, p. 247) says that sections may be transferred direct into Canada balsam, which is miscible with anise oil.

CHAPTER XI.

SERIAL SECTION MOUNTING.

185. Choice of a Method.—All the following methods are excellent if properly carried out. I recommend for general work the following:—For paraffin sections that have been already stained, Schällibaum's collodion. For paraffin sections that are to be stained on the slide, Mayer's albumen, unless the stain to be employed be one that will stain the albumen or if the sections be badly folded, in which case take one of the water or alcohol methods given in the next section. For collodion sections, Mayer's albumen. For very large collodion sections, Weigert's process.

Methods for Paraffin Sections.

186. The Water or Alcohol Method.—The principle of this method is due to GAULE (*Arch. f. Anat. u. Phys.* [*Phys. Abth.*], 1881, p. 156), who practised it as follows:—A slide is moistened with alcohol, the sections are arranged on it by means of a camel-hair brush, also moistened with alcohol; the slide is slightly warmed so as to cause the sections to stick to the slide; a cover is put on; the excess of paraffin is removed by means of a drop of pure xylol, and the mount is completed by means of xylol balsam.

Both the moistening with alcohol and the heating are necessary for the attachment of the sections to the slide; the effect is not obtainable by means of one of these manœuvres alone.

In the primitive form given above, Gaule's method is only applicable to the purpose of mounting a small series of sections that do not require to be stained or otherwise further manipulated on the slide. Later workers have by improvements in the details of the process brought it to a state of considerable perfection, so that it may now be said to be a fairly safe process for extensive series of sections, and will allow of staining on the slide.

SUCHANNEK (*Zeit. f. wiss. Mik.*, vii, 4, 1891, p. 464) pointed out that the slides must be *absolutely free from grease* in order that the alcohol (50 per cent.) or distilled water, which may be used instead, may spread out in a thin and uniform layer. Secondly, that the slides *should not be warmed to* *more than* 40° C., it being important both that *the alcohol should evaporate slowly* and that *the paraffin should only be softened, not melted,* until the evaporation is complete. HOYER had already for this reason advised slow evaporation at the temperature of the laboratory.

GULLAND (*Journ. of Anat. and Physiol.*, xxvi, 1891, p, 56; *Journ. Roy. Mic. Soc.*, 1892, p. 161) floats sections on to the surface of warm water (not warm enough to melt the paraffin), or alcohol if preferred, in a dish, and thence floats them into position on the slide. The slide is drained, and the water evaporated from it at a low temperature as described above. When the water of the sections has evaporated completely they become more transparent, and look dry. The fixation is then complete, the paraffin may be melted and removed by means of any desired solvent, and the sections may be mounted, or be stained in any medium, or otherwise manipulated as desired. Thin sections will generally be fixed in about an hour; thick ones will require six hours or more.

Here it may be pointed out that the degree of adhesion seems to depend very much on the nature of the sections. SCHIEFFERDECKER (*Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 202) finds that the larger and thinner sections are, the better do they stick, and *vice versâ*. And Mr. ANDREW PRINGLE writes me that he finds that tissues thoroughly fixed in chrome solutions, so that their albuminoid substances have become quite insoluble, do not adhere sufficiently without the aid of some substance to fix them to the slide. He prefers arranging sections on the slide with cold water, and then warming until the sections flatten out.

M. HEIDENHAIN (*Kern und Protoplasma*, p. 114, in "Festschr. f. Kölliker," 1892) also proceeds in this way, and advises that the heating be not carried to above 35° C., at which temperature several hours at least are necessary to ensure fixation. He also finds that water is preferable to alcohol, which is too mobile on the slide and evaporates too quickly. DURHAM's method (*Quart. Journ. Mic. Sci.*, xxxiii, 1891, p. 116; *Journ. Roy. Mic. Soc.*, 1892, p. 293) is the same as the last described, with the exception that he uses 70 per cent. alcohol instead of water.

WLASSAK (MERCIER's *Coupes du Système Nerveux Central*, 1894, p. 118) places the sections first of all on a drop of warm water on the slide, then exposes them to vapour of boiling distilled water, and then puts them in the stove.

The advantage of the water or alcohol method is that as there is nothing on the slide that can stain in plasma stains, it gives very clean preparations. The disadvantages are that, besides being excessively lengthy, it is *certainly not safe for chrome-osmium preparations.*

187. Schällibaum's Collodion Method (*Arch. f. mik. Anat.*, 1883, p. 565).—One part of collodion is shaken up with three to four volumes (according to the consistency of the collodion) of clove oil or lavender oil. This should give a clear solution. A little is spread thinly on a slide with a small brush. After arranging the sections on the prepared surface, warm over a water-bath, gently, until the clove oil has evaporated (five to ten minutes). The sections are then found to be fixed, and can be treated for days with turpentine, chloroform, alcohol, and watery fluids, without becoming detached. The advantage of this method is that it allows of staining on the slide. If after staining any cloudiness should appear between the sections, dehydrate the slide and treat it several times with absolute alcohol and turpentine, warming it gently the while; or brush the space between the sections repeatedly with a brush moistened with clove oil. This cloudiness only arises from the collodion solution having been taken too concentrated, or having been laid too thick on the slide.

I find it is not necessary to evaporate over a water-bath. It is sufficient to hold the slide over a spirit lamp until the paraffin has melted and the clove oil has collected in drops between the sections. Schällibaum has stated elsewhere that long evaporation of the slide is necessary if the sections are to be secured firmly enough to allow of staining on the slide. That is not so. What is necessary is that the paraffin and clove oil be thoroughly removed from contact with the sections; and that can be done in a second by melting and blowing the paraffin and oil away as described in § 140.

Rabl (*Zeit. f. wiss. Mik.*, xi, 2, p. 179) says that new solution of Schällibaum, not more than four or five days old, will stick fast enough to resist absolute alcohol. He gives the proportions of three parts of clove oil to two of collodion.

Personally I do not consider Schällibaum's method so safe as Mayer's albumen (and some other methods) for objects that are to be stained on the slide. I recommend it for already stained objects, because it is found to work very pleasantly. Its great defect is that it does not readily lend itself to any device for the flattening out of folded sections. I recommend xylol or naphtha for clearing, in preference to turpentine.

Field and Martin (*Bull. Soc. Zool. de France*, 1894, p. 48) say that treatment with xylol, toluol, or benzin makes collodion more readily soluble in alcohol, and recommend for clearing after Schällibaum's fixative either petroleum ether (a light petroleum, density 0·650) or chloroform.

Gallemaerts (*Bull. Soc. Belge de Micro.*, xv, 1889, p. 56;

Zeit. f. wiss. Mik., vi, p. 4, 1889, p. 493), following Drash, employs a saturated solution of gun-cotton in acetone, diluted to the requisite thinness with absolute alcohol.

Gage prefers preparing slides with a layer of pure collodion, which is allowed to dry, and is rendered adhesive at the instant of using by brushing with clove oil.

Summers (*Amer. Mon. Mic. Journ.*, 1887, p. 73; *Zeit. f. wiss. Mik.*, iv, 4, 1887, p. 482) also employs a dry layer of collodion, which he renders adhesive after the sections are arranged on it, by wetting with a mixture of equal parts of alcohol and ether. As soon as the mixture has evaporated, the sections are found to be fixed.

Good collodion is essential in this process.

Strasser (*Zeit. f. wiss. Mik.*, iv, 1, 1887, p. 45) recommends a mixture of 2 parts collodion, 2 parts ether, and 3 parts castor oil; or (*ibid.*, vi, 2, 1889, p. 153) 2 parts of collodion with one of castor oil, the sections being painted over with a thicker solution, viz. collodium concentratum duplex 2 to 3 parts, castor oil 2 parts, and the slide being plunged at once, without warming, into a bath of turpentine, in which it remains till the paraffin is dissolved (two to ten hours, somewhat less if the whole be put in a stove). The turpentine suffices to harden the collodion (benzin, benzol, and chloroform have the same effect).

188. Strasser's Collodion-Paper Method (*Zeit. f. wiss. Mik.*, iii, 3, 1886, p. 346).—This is an extremely complicated modification of Weigert's method for celloidin sections, and is only adapted for use with Strasser's automatic ribbon-microtome. See the original papers in *Zeit. f. wiss. Mik.*, iii, 3, 1886, p. 346; vi, 2, 1889, p. 154; vii, 3, 1890, p. 290; ib., p. 304; and ix, 1, 1892, p. 8; see also a very short abstract of the last paper in *Journ. Roy. Mic. Soc.*, 1892, p. 703, in which is a figure of the "Schnitt-Aufklebe-Mikrotom;" and *Zeit. f. wiss. Mik.*, xii, 2, 1895, p. 154 (*Journ. Roy. Mic. Soc.*, 1895, p. 702).

189. The Shellac Method (Giesbrecht, *Zool. Anz.*, 1881, p. 484).—Prepare a stock of slides covered with a thin and even film of shellac. This is done as follows:—Make a not too strong solution of brown shellac in absolute alcohol, filter it thoroughly; warm the slides, and spread over them a layer of shellac by means of a glass rod dipped in the solution and drawn once over each slide. Let the slides dry.

Just before beginning to cut your sections take a prepared slide and brush it over *very thinly* with kreasote applied by means of a brush; this forms a sticky surface on which the sections are now arranged one by one as cut, care being taken to bring them on to the slide with as little surrounding paraffin as possible.

When all the sections are arranged the slide is heated on a water-bath for about a quarter of an hour at the melting-point of the paraffin. The slide is allowed to cool, and the sections are now found to be firmly fixed in the shellac. The paraffin is dissolved away by dropping turpentine on to the sections, which are then mounted in Canada balsam. There is no danger of the sections being floated away by the turpentine, because turpentine does not dissolve shellac.

In the note in the *Zool. Anz.* above quoted, the shellac solution is stated to be prepared with common brown shellac (choosing, of course, by preference the paler sorts), on account of the insolubility of white shellac in alcohol. In the *Mitth. d. Zool. Stat.* of Naples, of the same year, "bleached white shellac" is recommended to be dissolved as before in absolute alcohol. In the *Journ. Roy. Mic. Soc.* (N.S.), vol. ii, 1882, p. 888, it is stated (on whose authority is not clear) that the solution is made by mixing 1 part of bleached shellac with 10 parts absolute alcohol, and filtering. In the same place it is added that "Dr. Mark uses the bleached shellac in the form in which it is prepared for artists as a 'fixative' for charcoal pictures."

The account given in the *Mitth. d. Zool. Stat.* further varies in one other detail from that given in the *Zool. Anz.* It directs that the shellac slides be brushed before cutting with *oil of cloves* instead of kreasote, the slide being slightly warmed before brushing.

The white shellac of commerce is sometimes not easily soluble in alcohol. Kingsley (see Whitman's *Methods in Microscopical Anat.*, p. 117) recommends that brown shellac be taken, and bleached by exposure to the sun.

Caldwell (*Quart. Journ. Mic. Soc.* [N.S.], lxxxvii, 1882, p. 336) simplifies the method by merely brushing over the side (thinly) at the moment of using with a strong solution of shellac in anhydrous kreasote. (To make the solution, warm the kreasote.)

In both the foregoing methods it often happens that the shellac becomes granular or cloudy on the slide. P. Mayer attributes this to the kreasote or clove oil, and proposes to remedy it by employing carbolic acid instead (*Amer. Natural.*, 1882, p. 733; *Zeit. f. wiss. Mik.*, iv, 1, 1887, p. 77; *Journ. Roy. Mic. Soc.*, 1885, p. 910). Powdered white shellac is heated with crystallised carbolic acid till it dissolves, and the solution filtered warm.

But more recently (*Intern. Monatschr. f. Anat., &c.*, 1887, Heft 2; *Zeit. f. wiss. Mik.*, iv, 1, 1887, p. 77) the same author, on the ground that hot carbolic acid attacks some tissues, recommends another method. Slides are prepared with alcoholic shellac according to Giesbrecht's plan. The sections are arranged on the dry film and gently pressed down on to it, then exposed for half a minute to vapour of ether.

Chloroform softens shellac; therefore chloroform balsam is not a safe mounting medium for sections fixed by these methods.

These methods do not allow of staining on the slide.

I feel bound to say that I am at a loss to understand by what virtue it is that the shellac method continues to survive, as it certainly seems to do, in the face of far more convenient and efficient processes.

REESE UNIV

190. Mayer's Albumen (*Mitth. Zool. Stat. Neapel*, iv, 1883; *Journ. Roy. Mic. Soc.* [N.S.], iv, 1884, p. 317; *Internat.*

Monatschr. f. Anat., 1887, Heft 2; *Journ. Roy. Mic. Soc.*, 1888, p. 160).—White of egg, 50 c.c.; glycerin, 50 c.c.; salicylate of soda, 1 grm. Shake them well together, and filter into a clean bottle. The filtering may take days or a week, but the preparation does not spoil meanwhile.

FOL (*Lehrb.*, p. 134) takes whipped white of egg, filters it through a Bunsen filter, and adds the glycerin and a little camphor or carbolic acid.

I find it convenient to beat up the egg with a little water before adding the glycerin and filtering, the salicylate being dissolved in the water in the first instance. But in view of the good preservation of the mixture it is perhaps better not to add water; the salicylate will dissolve without it with sufficient shaking.

According to my experience carbolic acid is perfectly efficient as a preservative, but is not to be recommended because it precipitates a great deal of the albumen.

A thin layer of the mixture is spread on a cold slide with a fine brush, and the sections laid on it and warmed for some minutes on a water-bath. (*Press the sections well down into the albumen with a brush.*) As the paraffin in the sections melts it carries the albumen away from them, and this is one of the advantages of the method. The sections may be treated with turpentine, alcohol, and aqueous or other stains without any danger of their moving.

It is not necessary to use a water-bath for warming the slide. I prefer to warm for an instant over a flame until the paraffin melts, and then blow away the melted paraffin as described, § 140. The remaining paraffin is instantly removed by means of xylol, toluol, or the like.

It is not *necessary* to warm the slide at all; *the paraffin can be removed in the cold if desired* by putting the slide into toluol, xylol, or the like. But the slide should be very thoroughly treated with alcohol after removal of the paraffin, in order to get rid of the glycerin, which will cause cloudiness if not perfectly removed.

The function of the glycerin is merely to keep the layer of albumen moist.

Miss A. M. CLAYPOLE has written a paper complaining that the method is uncertain, because too much heat may injure the tissues, and if too little be applied the albumen will not coagulate. This is a misapprehension. No heat whatever is required to coagulate the albumen; the alcohol will do that sufficiently. The complications proposed by Miss Claypole are not only unnecessary but undesirable. The only object in using heat is to enable one to get rid of the paraffin quicker; and it cannot be supposed that tissues that have been for perhaps several hours in a bath of paraffin can suffer through being exposed for a single instant more to the temperature required to melt away the same paraffin from the sections.

This method allows of the staining of sections on the slide with perfect safety, both with alcoholic and aqueous stains.

This method can be combined with the water process for flattening out sections (§ 186), as described by HENNEGUY (*Journ. de l'Anat. et de la Physiol.*, 1891, p. 398). A drop of water is spread by means of a glass rod on a slide prepared with white-of-egg mixture, the sections are arranged on it, the whole is warmed (*not* to the melting-point of the paraffin) until the sections flatten out; the water is then evaporated off at a temperature of about 40° C., and as soon as it has entirely disappeared the paraffin is melted, and the slide further treated as above described.

See also the careful and detailed description of this method given by OHLMACHER (*Journ. Amer. Med. Ass.*, April, 1893), who has independently worked out the same process.

According to my experience the albumen method is *absolutely safe*, and is the one that should in general be preferred for staining on the slide. It has the defect that certain plasma-stains (not chromatin stains) colour the albumen very strongly and cannot be removed from it. This produces very ugly mounts.

It sometimes happens that the mixture after it has stood for some time becomes turbid, a change which is attributed to the development of a microbe. I know of no means of preventing the mixture from going bad in this way, though I have found that it keeps better when freely exposed to the sun. It has been stated (VOSSELER, *Zeit. f. wiss. Mik.*, vii, 4, 1891, p. 457) that as soon as the mixture has become turbid it loses its adhesive properties and should be thrown away. That is not my experience. I find the liquid first becomes milky, then altogether turbid, and at last coagulates, passing into a caseous state. But up to the very last it does not in the least degree lose its adhesive properties. As long as there is enough moisture in it to moisten the brush, it will stick as well as the first day.

191. Mann's Albumen Method (*Zeit. f. wiss. Mik.*, xi, 4, 1894, p. 486).—Shake up white of egg with ten volumes of distilled water and filter twice through the same paper. Spread this on a stock of slides with a glass rod, let them drain and dry. Float the sections on to water warmed to 40° C., pass a slide beneath them, arrange them in order, lift them out, put the slide for five minutes on a stove heated to 35° C., then treat with xylol and alcohol.

192. Flögel's Gum Method (*Zool. Anz.*, 1883, p. 565).—Make a solution of one part gum arabic in twenty parts water; filter, and add a little alcohol to prevent the formation of mould. Slides are prepared by pouring the solution over them, and draining. (It is important that the slides be so perfectly clean as to be evenly wetted all over by the gum solution.) Sections may now be cut and laid on the gum surface before it has become dry, and floated into the proper position; this is the best plan for sections of $\frac{1}{100}$ mm. thickness, and for large sections. For thinner and small sections it is best to take slides that have completely dried, arrange the sections dry on the gum film, and then breathe on it until the gum has become sticky.

A very neat method for cases in which it is not required to treat the slide with watery fluids.

WADDINGTON (*Journ. Quek. M. Club*, vi, 1881, p. 199; *Journ. Roy. Mic. Soc.* [N.S.], i, 1881, p. 704) gives the following process for preparing "arabin," a purified gum arabic which has the advantage of not presenting a granular appearance under the microscope as ordinary gum arabic does.

Dissolve clear and white gum arabic in distilled water to the consistency of thin mucilage. Filter. Pour the filtrate into rectified alcohol, and shake well; the arabin separates as a white pasty mass. Place it on filter-paper, and wash with pure alcohol until the washings are free from water. Dry.

The white powder thus obtained should be dissolved in distilled water and filtered twice. It may then be placed on slides, which are drained, dried, and put away till wanted. In this condition it may be preserved indefinitely.

193. Frenzel's Gum Method (*Arch. f. mik. Anat.*, Bd. xxv, 1885, p. 51).—Gum arabic is dissolved in water to the consistency of a thin mucilage, and to this is added aqueous solution of chrome-alum. An excess of the latter does no harm. Finally add a little glycerin and a trace of alcohol (l. c., p. 142). The slide is prepared with this in the usual way; the sections (either cut dry or in the wet way) are gently pressed on to it with a brush and slightly melted on, and heated for at most a quarter of an hour at a temperature of 30° to 45° C., which suffices to render the gum insoluble. This layer has the advantage of not staining with the majority of staining fluids; fuchsin and safranin are the only ones that stain it to a harmful degree. In the other anilins, and in carmine or hæmatoxylin, it does not stain. Watery stains (it is stated) may be used with it.

194. **Born and Wieger's Quince-Mucilage** (*Zeit. f. wiss. Mik.*, 1885, p. 346).—To two volumes of the ordinary pharmaceutical quince-mucilage (*Mucilago cydonii*) add one volume of glycerin and a trace of carbolic acid. Spread in a thin layer on a carefully cleaned slide, and arrange the sections on the moist surface. Heat for twenty minutes at a temperature of 30° to 40° C. After removal of the paraffin by turpentine the slide is brought for half an hour into *absolute* alcohol. You may then mount, or pass through successive alcohols, and stain. Alkaline staining fluids must be avoided, as they soften the mucilage and cause the sections to become detached.

195. GRAVIS's **Agar-agar** (*Bull. Soc. Belge de Micr.*, xv, 1889, p. 72; *Zeit. f. wiss. Mik.*, vi. 4, 1889, p. 494).—Solution of agar-agar in 1000 parts of water, to be used as in last section.

196. GRAY's **Gelatin Process** (*The Microscope*, ix, 1889, p. 325; *Journ. Roy. Mic. Soc.*, 1890, p. 117).—Solution of gelatin in 100 parts of water. Use as gum arabic solution, taking care not to melt the paraffin; let the slide dry spontaneously overnight, and remove the paraffin with a suitable solvent; remove the solvent with alcohol, and then treat for five minutes with 2 per cent. solution of potassium bichromate to render the gelatin insoluble. Stain as desired, or mount.

HENNEGUY (*Leçons sur la Cellule*, p. 62) takes a solution of gelatin of about 1 : 5000, to which a trace of bichromate of potash is added at the instant of using. The sections are flattened out by warming, the slide is drained, and dried for some hours exposed to the light.

ALLEGER (*Proc. Amer. Mic. Soc.*, xv, 104 and 192; quoted from FISH, ibid., xvii, 1895, p. 321) uses formalin in much the same way. "A few drops of formalin are added to each gramme of a 0·5 to 1 per cent. gelatin solution. A gentle heat is applied to the slide until the paraffin is softened, and the superfluous gelatin allowed to drain from the edge of the slide."

EISEN (*Proc. Cal. Acad. Sci.*, v, 1895, p. 4; *Journ. Roy. Mic. Soc.*, 1895, p. 486) practises the same process, explaining that "the fixing should be allowed to harden in the air for at least four hours, or better during the night," after the sections have been arranged on it.

197. VAN WALSEM's **Gelatin Process.**—Extremely complicated. See the six pages of description in the original, *Zeit. f. wiss. Mik.*, xi, 2, 1894, pp. 229 to 235, or the abstract in *Journ. Roy. Mic. Soc.*, 1895, p. 122.

198. OBREGIA's process given below, for celloidin sections, is also applicable to paraffin sections.

Methods for Watery Sections.

199. Fol's Gelatin (FOL, *Lehrb.*, p. 132).—Four grammes of gelatin are dissolved in 20 c.c. of glacial acetic acid by heating on a water-bath and agitation. To 5 c.c. of the solution add 70 c.c. of 70 per cent. alcohol and 1 to 2 c.c. of 5 per cent. aqueous solution of chrome-alum. Pour the mixture on to the slide and allow it to dry. In a few hours the gelatin passes into the insoluble state. It retains, however, the property of swelling and becoming somewhat sticky in presence of water. The slide may then be immersed in water containing the sections; these can be slid into their places, and the whole lifted out: the sections will be found to be fixed in their places.

This method is especially useful for sections made under water, large celloidin sections amongst others.

200. POLI (*Malpighia*, ii, 1888, 2, 3; *Zeit. f. wiss. Mik.*, v, 3, 1888, p. 361) arranges sections on a layer of melted Kaiser's gelatin, adds glycerin, and covers. See also *supra*, § 196.

201. Frenzel and Threlfall's Gutta-percha (or Caoutchouc) Method (*Zool. Anz.*, 1883, pp. 51, 301, and 423).—This extremely elegant method is not perfectly safe, the gutta-percha film being liable to tear; and is now, I believe, very generally abandoned.

Methods for Celloidin Sections.

202. The Albumen Method.—I find that celloidin sections may be mounted on Mayer's albumen, and have the celloidin removed if desired by putting them into ether-alcohol. Care must be taken to press them down very thoroughly on to the albumen.

203. SUMMER's **Ether Method** (*Amer. Mon. Mic. Journ.*, 1887, p. 73; *Zeit. f. wiss. Mik.*, iv, 4, 1887, p. 482; *Journ. Roy. Mic. Soc.*, 1887, p. 523).—Besides the method given above (§ 187), which is applicable to celloidin sections, but is needlessly complicated, Summers recommends the following simpler method:—Place the sections in 95 per cent. alcohol for a minute or two, arrange on the slide, and then pour over the sections sulphuric ether *vapour*, from a bottle partly full of liquid ether. The celloidin will immediately soften and become perfectly transparent. Place the slide in 80 per cent. alcohol, or even directly in 95 per cent. if desired. The sections, it is said, will be found to be firmly fixed, and may be stained if desired. I have myself not found this method safe.

SCHIEFFERDECKER (*Zeit. f. wiss. Mik.*, v, 4, 1888, p. 507) recommends that the slide be one that has been previously prepared with a layer of collodion if it is desired to stain on the slide; but if not a clean slide is perfectly sufficient. The slide may of course be treated with ether vapour in a preparation glass or similar arrangement.

GAGE (*Proc. Amer. Soc. Mic.*, 1892, p. 82) advises that the slide be one that has been previously coated with a 0·5 per cent. solution of gelatin and dried; the collodion adheres much more strongly to a gelatinised surface.

204. EYCLESHEIMER's **Method** (*Amer. Natural.*, xxvi, 1892, p. 354; *Journ. Roy. Mic. Soc.*, 1892, p. 565).—The sections are arranged on a slide with enough alcohol to keep them moist, but not enough to float them. They are covered with a strip of toilet paper, which is kept down in place by winding thread round the slide. Care should be taken to have the turns of the thread passing between the sections, not over them. You may now stain and clear in any way that may be desired. After clearing, you cut the thread, remove the paper by rolling it up from one end, and mount.

205. APÁTHY's **Oil of Bergamot Method** (*Mitth. Zool. Stat. Neapel*, 1887, p. 742; *Zeit. f. wiss. Mik.*, v, 1, 1888, p. 46, and v, 3, 1888, p. 360; *Journ. Roy. Mic. Soc.*, 1888, p. 670). —Cut with a knife smeared with vaselin (§ 162) and wetted with 95 per cent. alcohol. Float the sections, as cut, on bergamot oil (must be green, must mix perfectly with 90 per cent. alcohol, and must not smell of turpentine). The sections spread themselves out on the surface of the oil; before they sink, each one is pushed by means of a needle into its place on a slip of tracing-paper dipped into the oil. (A good size for the paper is about as broad as the slide, and three times as long as the cover.) When the requisite number of sections have been arranged on the paper, you drain the paper, dry the under side of it with blotting-paper, turn it over, and gently press it down with blotting-paper on to a carefully dried slide. Remove the paper by rolling it up from one end. The sections remain adhering to the slide, and may have the remaining bergamot oil removed from them by means of a cigarette paper. If they are already stained, nothing remains but to add balsam and a cover.

In the case of unstained or very small objects, it is well to add a little alcoholic solution of safranin to the bergamot oil. The celloidin of the sections becomes coloured in it in a few seconds, and makes them readily visible. The colour disappears after mounting in a few days.

If the sections are to be stained, the slide after removal of the bergamot oil is exposed for a few minutes to the vapour of a mixture of ether and alcohol, then brought into 90 per cent. alcohol, and after a quarter of an hour therein may be stained in any fluid that contains 70 per cent. alcohol or more.

If it be desired to stain in a watery fluid, care must have been taken when arranging the sections to let the celloidin of each section overlap that of its neighbours at the edges, so that the ether vapour may fuse them all into one continuous plate. This will become detached from the slide in watery fluids, and may then be treated as a single section.

206. Apáthy's **Series-on-the-Knife Method** (*Zeit. f. wiss. Mik.*, vi, 2, 1888, p. 168).—The following is in some respects more convenient than the oil of bergamot method. The knife is well smeared with yellow vaselin rubbed evenly on with the finger, and is wetted with alcohol of 70 to 90 per cent. As fast as the sections are cut they are drawn with a needle or small brush to a dry part of the blade, and there arranged in rows, the celloidin of each section overlapping or at least touching that of its neighbours. The rows are the length of the cover-glass, and are arranged one under the other so as to form a square of the size of the cover-glass. When a series (or several series, if you like) has been thus completed, the sections are dried by laying blotting-paper on them (there is no risk of their becoming attached to it, as they are held down by the vaselin). The series is then painted over with some of the thickest celloidin solution used for imbedding, is allowed to evaporate for five minutes in the air, and is then either wetted with 70 per cent. alcohol, and allowed to remain whilst cutting is proceeded with, or (if no more sections are to be cut, or if the knife is now full) the knife is removed and brought for half an hour into 70 per cent. alcohol. This hardens the celloidin around the sections into a continuous lamella, which can be easily detached by means of a scalpel, and stained, or further treated as desired. It is well to bring it at once on to a slide, moisten the edges of the celloidin plate with ether and alcohol mixture, so that it may not become detached, and bring the whole into the staining solution.

207. WEIGERT'S **Collodion Method** (*Zeit. f. wiss. Mik.*, 1885, p. 490).—Sections are cut wet with alcohol. Care should be taken not to have so much alcohol on the knife as to cause the sections to float. Prepare a slip of porous but tough paper (Weigert recommends "closet paper"), of about twice the width of the sections. Soak it in alcohol, take it by both ends, stretch it slightly, and lower it on to the section that is on the knife. The section will adhere to the paper, and is taken up by moving the slip horizontally or slightly upwards, away from the edge of the knife. Take up the first section towards the end of the paper that you hold in your left hand, and let the remaining sections follow in order from left to right. After each section has been taken up, the slip is placed, whilst the next section is being cut, with the sections upwards on a moist surface prepared by arranging several layers of blotting-paper, covered with one layer of closet paper, in a plate, and saturating the whole with alcohol. When all the sections have been arranged on the slip, you pass to the next stage of the process, the collodionisation of the series.

This is done in two steps. The first of these consists in transporting the series on to a plate of glass prepared with collodion. The plate is prepared beforehand by pouring on to it collodion and causing it to spread out into a thin layer, as photographers do, and allowing it to dry. (A number of the plates may be prepared and kept indefinitely in stock; microscope slides will do for series of small sections.) Take one of these plates; lay the slip of paper with the sections on the plate, the sections downwards; press it down gently and evenly, and the sections will adhere to the collodion, then carefully remove the paper. (Do not place more than one or at most two lines of sections on the same plate, for those first placed run the risk of becoming dry whilst you are placing the others.) This finishes the first stage of the collodionising process.

Now remove with blotting-paper any excess of alcohol that may remain on or around the sections, pour collodion over them, and get it to spread in an even layer. As soon as this layer is dry at the surface you may write any necessary indications on it with a small brush charged with methylen blue (the colour will remain fast throughout all subsequent manipulations).

The plate may now either be put away till wanted in 80 per cent. alcohol, or may be brought into a staining fluid. Weigert recommends his hæmatoxylin process, but other watery stains may be used. The watery fluid causes the double sheet of collodion to become detached from the glass, holding the sections fast between its folds. It is then easy to stain, wash, dehydrate, and mount in the usual way, merely taking care not to use alcohol of more than 90 to 96 per cent. for dehydration. Weigert recommends for clearing the above-described mixture of xylol and carbolic acid (§ 164). Both the dehydration and the clearing take rather longer with the collodionised series than with free sections.

The series should be cut into the desired lengths for mounting whilst in the alcohol. It is perhaps safer to lay them out for cutting on a strip of closet paper saturated with alcohol.

It is hardly necessary to comment on the great value of this beautiful method.

It is suggested by Strasser that gummed paper might be an improvement on the glass plates used in this process—especially for very large sections. See *ante*, § 187.

The modification of Weigert's method proposed by Wintersteiner (*Zeit. f. wiss. Mik.*, x, 3, 1893, p. 316) consists in suppressing the alignment of the sections on the strip of paper, and slipping them direct from the knife on to the prepared glass.

208. Obregia's **Method.**—This method was originally described in the *Neurologisches Centralb.* for 1890, and is given in the third edition of Woodhead's *Practical Pathology.* It has been recommended for class purposes as being very safe and convenient by Gulland (*Journ. of Path.*, February, 1893). Slides, or glass plates of any size, are coated with a solution made of—

Syrupy solution of powdered candy-sugar made with boiling distilled water	30 c.c.
Absolute alcohol	20 ,,
Transparent syrupy solution of pure dextrin made with distilled water .	10 ,,

They are dried slowly for two or three days until the surface is just sticky to the moist finger. Sections are arranged and heated for a few minutes to a temperature slightly above the melting-point of the paraffin. The paraffin is removed by some solvent, such as xylol or naphtha, and this is in turn removed by alcohol. The alcohol is poured off, and the sections are covered with solution of celloidin or with a solution of 3 per cent. of photoxylin in a mixture of equal parts of ether and absolute alcohol. The plates are left to evaporate in a horizontal position, and when the sections are required the sheet of collodion is cut into ribbons, which are floated off in water, and further treated as desired, *e.g.* as in Weigert's process, § 207. (It is well to divide the sheet of collodion into ribbons by running the point of a knife down it as soon as evaporation has produced a very slight solidification, and the evaporation must not be artificially hastened.)

This is the process for paraffin sections; for celloidin sections the sections are taken up in order on a strip of paper as in WEIGERT's method, and laid down on the glass in the same way, and then covered with the photoxylin solution and evaporated as described. The advantage of Obregia's process is that it is equally applicable to paraffin sections, to celloidin sections, and to sections of material that has not been imbedded at all.

209. GIACOMINI's collodion-gelatin process for large sections, see *Gazzetta delle Cliniche*, November, 1885, *Zeit. f. wiss. Mik.*, 1885, p. 531, or the first edition of the *Traité* of LEE et HENNEGUY, p. 392.

CHAPTER XII.

STAINING.

210. The Kinds of Stains.—Stains are either General or Special (otherwise called Specific, or Selective, or Elective). A general stain is one that takes effect on all the elements of a preparation. A special, specific, selective, or elective stain is one that takes effect only on some of them, certain elements being made prominent by being coloured, the rest either remaining colourless or being coloured with a different intensity or in a different tone. To obtain this *differentiation* is the chief object for which colouring reagents are employed in microscopic anatomy.

Two chief kinds of this selection may be distinguished,—histological selection and cytological selection. In the former an entire tissue or group of tissue-elements is prominently stained, the elements of other sorts present in the preparation remaining colourless or being at all events differently stained, as in a successful impregnation of nerve-endings by means of gold chloride. This is the kind of stain that is generally meant by a *specific* stain. In the latter the stain seizes on one of the constituent elements of cells in general, namely, either the nucleus or the extra-nuclear parts.

Stains that thus exhibit a selective affinity for the substance of nuclei—*nuclear* or *chromatin* stains—form at present by far the most important class of stains—in zootomy at any rate. What the zootomist wants, and the histologist too, in the great majority of cases, is either to differentiate the intimate structures of cells by means of a colour reaction, in order to study them for their own sakes, or to have the nuclei of tissues marked out by staining in the midst of the unstained material in such a way that they may form landmarks to catch the eye, which is then able to follow out with ease the contours and relations of the elements to which the nuclei belong; the extra-nuclear parts of these elements being expressly left unstained in order that as little light as possible may be absorbed in passing through the preparation. Possibly this may be an irrational procedure, but it has hitherto been found in practice to be the most efficient for general work.

To these two kinds of selective stains must be added a third group, the *plasmatic* stains, consisting of those few stains that take effect in a special way on cytoplasm, or formed tissue or ground-substance, leaving the chromatic substance of nuclei as far as possible unstained. In this book, therefore, stains are looked upon as being (1) General stains; (2) Selective stains; the latter group being subdivided into (*a*) Nuclear, (*b*) Plasmatic, (*c*) Histologically Selective, or Specific. This classification, however, is not followed in the arrangement of the special paragraphs, it being more practical to follow an order based on the chemical nature of the staining agents, and on convenience of exposition.

Some writers have divided stains into nuclear, general, and selective. This arrangement appears to me faulty because every nuclear stain is *eo ipso* selective, and because it ignores the subdivisions of selective stains.

211. The Methods of Staining.—Colouring matters possessing so great an affinity for certain elements of tissues that they may be left to produce the desired electivity of stain without any special manipulation on the part of the operator, are unfortunately rare. In practice, selective staining is arrived at in two ways. In the one, which may be called the *progressive* or *direct* method, you make use of a colouring reagent that stains the element desired to be selected more quickly than the elements you wish to have unstained; and you stop the process and fix the colour at the moment when the former are just sufficiently stained, and the latter not affected to an injurious extent, or not affected at all, by the colour. This is what happens, for instance, when you stain the nuclei of a preparation by treatment with very dilute hæmatoxylin: you get, at a certain moment, a fairly pure nuclear stain; but if you were to prolong the treatment, the extra-nuclear elements would take up the colour, and the selectivity of the stain would be lost. It may be noted of this method that it is in general the method of *fast* stains ("echte Farbung"), and that it renders great services in the colouring of specimens *in toto*,—a procedure which is not possible with the chief stains of the other class (the anilins). It is the old method of carmine and hæmatoxylin staining.

The second, the *regressive* or *indirect* method, is the method of overstaining followed by partial discoloration. You begin by staining all the elements of your preparation indiscriminately, and you then wash out the colour from all the elements except those which you desire to have stained, these retaining the colour more obstinately than the others in virtue of a certain not yet satisfactorily explained affinity. This is what happens, for instance, when you stain a section of one deep red in all its elements with safranin, and then treating it for a few seconds with alcohol, extract the colour from all but the chromatin and nucleoli of the nuclei. It is in this method that the coal-tar colours find their chief employment. It is in general applicable only to sections, and not to staining objects *in toto* (the case of borax-carmine is probably only a seeming exception to this statement). It is a method, however, of very wide applicability, and gives, perhaps, the most brilliant results that have hitherto been attained.

In previous editions the expressions "direct" and "indirect" staining methods were alone used. The expressions "progressive" and "regressive" are due to M. HEIDENHAIN, and appear to me to be preferable.

212. The State of the Tissues to be Stained.—It is generally found that precise stains can only be obtained with carefully fixed (*i. e.* hardened) tissues. Dead, but not artificially hardened tissues stain indeed, but not generally in a precise manner. Living tissue elements in general do not stain at all, but resist the action of colouring reagents till they are killed by them (see, however, next section).

It appears probable, as was first pointed out, I believe, by PAUL MAYER, that the usual histological stains obtained with fixed tissues are brought about in two ways. Either they result from the combination of the colouring agent with certain organic or inorganic salts,—phosphates, for instance, that existed in the tissue elements during life and were thrown down *in situ* by the fixing or hardening agent employed, as seems to happen when such a fixing agent as alcohol is employed. Or they result from the combination of the colouring agent with certain compounds that did not pre-exist in the tissues, but were formed by the combination of the constituents of the tissues with the chemical elements brought to them by the fixing agent, as seems to happen when such a fixing agent as chromic acid is employed—the compounds in question being probably chiefly metal albuminates. These considerations will serve to show to how great an extent the quality of a stain is dependent on the nature of the previous treatment the tissues have undergone.

213. Staining "intra vitam."—Some few substances possess the property of staining—or rather, tingeing—living cells without greatly impairing their vitality. Such are—in very dilute solutions—cyanin (or quinoleïn), methylen blue, Bismarck brown, anilin black, and, under certain conditions, dahlia and eosin, gentian violet, with perhaps methyl violet, and some others whose action is not yet sufficiently established by experiment. Congo, even in strong solution, is not toxic to some organisms, and stains some structures (see SCHOLTZ, *Centralb. f. d. med. Wiss.*, 1886, p. 449; also *Journ. Roy. Mic. Soc.*, 1886, p. 1092). Living Rotifera are in part successfully stained by it during life. (The paper of Martinotti, *Zeit. f. wiss. Mik.*, v, 3, 1888, p. 305, may be consulted on this subject.) More recently, neutral red (*Neutralroth*) has been greatly recommended by Ehrlich (see Chap. XVI).

As to the employment of these reagents, it may be noted that they must be taken in a state of extreme dilution, and in neutral or feebly alkaline solution—acids being of course toxic to cells. Thus employed, they will be found to tinge with colour the cytoplasm of certain cells during life (never, so far as I know, nuclear chromatin during life;—if this stain, it is a sign that death has set in). The stain is sometimes diffused throughout the general substance of the cytoplasm, sometimes limited to certain granules in it, which have been taken, perhaps without sufficient reason, to be identical with the granules of Altmann (Altmann's *Studien über die Zelle*, 1886).

Since the publication of the last edition I have made a considerable number of observations on this subject, and have come to the same conclusion as GALLEOTTI (*Zeit. f. wiss. Mik.*, xi, 2, 1894, p. 172), namely, that the so-called "intra vitam" stains are not true stains at all. The diffused coloration above mentioned appears always, if the cell that shows it have remained in a state of unimpaired vitality, to be due to simple absorption or imbibition of the colouring matter by the cell, not to a chemical combination of the colouring matter with any of the constituents of the cell. If a cell thus coloured be transported into a medium free from the colouring matter it will give up unchanged the colour it had imbibed, which seems to be a sufficient proof that the colouring matter had not entered into any chemical combination with the elements of the cell, but was simply held in a mechanical way in the interstices of its substance. If, on the other hand, there has been produced the above-mentioned coloration of certain granules or other cell-contents, it is possible that this may be a true stain in the sense of being a chemical combination. It may be so, but it certainly is not always so, as may sometimes be proved with the greatest ease by putting the cell into a colourless medium and observing the supposed stain disappear. And in cases in which this does not happen, in which therefore a more or less fast stain has been obtained, it is invariably found that the stain is limited to cell-contents that do not form an integral part of the living texture of the cell; the cell itself may be living, but they are not. These granules or other cell-contents may be granules formed of substances that have been absorbed by the cell from without—food-granules; or they may be katabolic products, consisting of matter that is no longer alive and is destined to be shortly expelled from the cell; or they may be elements that form indeed an integral part of the living texture of the cell but have been injuriously affected by the colouring matter, and for that or some other reason are in a state of diminished vitality,—they are parts of the cell that are being killed by the colouring reagent or that have been totally killed by it whilst the rest survives; in no case do they consist of matter that is fully and perfectly alive. I am inclined to think that the chief scientific value of the so-called vital or *intra-vitam* stains will be found to lie in the fact that they may furnish us with the means of distinguishing the living constituents of a cell from the non-living ones, and even of recognising amongst the living ones those that possess only a relatively low or impaired degree of vitality.

Apart, however, from the question whether the elements stained by the so-called "vital" stains are truly living or not, it must be conceded that this mode of treating living cells has frequently a measure of practical utility. It often enables us to map out physiological or morphological tracts that would otherwise be unrecognisable or less readily recognisable in the living state.

I have frequently found gentian, dahlia, and methylen blue, added to indifferent liquids, extremely useful in the examination of tissue-cells. Quinoleïn and Bismarck brown are well-known aids to the study of Infusoria. Methylen blue has a specific affinity for sensory nerves, and is an extremely important reagent (see *post*, Chap. XVI). According to my experience, methylen blue is the most generally useful of these stains. It has the valuable point that it is perfectly soluble in saline solutions, and may therefore be employed with marine organisms by simply adding it to sea water. The others are not thus soluble to a practical extent, but I find that gentian and dahlia become so if a trace of chloral hydrate —0·25 per cent. is ample enough—be added to the saline solution. Any of these reagents may be rubbed up with serum, or other "indifferent" liquid.

Methylen blue may be fixed in the tissues, and permanent preparations made, by one or other of the methods described in Chap. XVI. Bismarck brown stains may be fixed with 0·2 per cent. chromic acid or with sublimate solution (Mayer), and the preparations may be stained with safranin, care being taken not to expose them too long to the action of alcohol.

214. Substantive and Adjective Staining; Mordants.—In the industry of dyeing, colouring matters are divided into two classes, according to their behaviour with respect to the material to be dyed. Certain dyes are absorbed directly from their solution by the material immersed therein, and combine with it directly. In this case the material is said to be *substantively* dyed, and the colouring matter is called a *substantive* colouring matter.

Other dyes do not combine directly with the material to be acted on, but this material must first be charged with some substance known as a *mordant* (generally a metallic salt or hydrate) before it will combine with the colouring matter. These are known as *adjective* colouring matters.*

Animal tissues have in general a considerable affinity for colouring matters, taking them up directly from their solutions. In consequence, the great majority of histological stains are obtained by substantive staining of the tissues. Still, as has been already pointed out, it seems probable that many of the histological stains that are obtained without intentional mordanting of the tissues, should yet in strictness be attributed to the class of adjective stains. This would be the case whenever there is reason to suppose that the stain obtained results from a combination of the colouring matter with some metallic salt or hydrate that is not a constituent of the living tissue, but has been brought into it by the fixing or hardening reagents, these reagents playing the part of mordants though only intentionally employed for another purpose. This would appear to be the case with the stains, or some of them, obtained after fixation with corrosive sublimate, alum, salts of iron, of platinum, of palladium, of uranium, and, for certain tissue elements and certain colours, chromium. And further, the mordanting substance may not only be present unintentionally in the fixing or hardening agents, it may be present unintentionally, or with imperfect realisation of its import, in the staining solutions themselves. Such is presumably the part played by alum in many of the stains in which it figures as an ingredient. Iodine also plays in some staining processes a part which seems only explicable on the supposition that it acts as a mordant.

In some staining processes, however, mordants are intentionally resorted to in order to fix the stain. Mordanting has long been employed in some hæmatein staining processes, such as that of M. HEIDENHAIN. More lately it has been resorted to for staining with tar colours, as in the curious "inversion" process of RAWITZ. These processes will be explained in their respective paragraphs. Here it remains only to note of what sort are the advantages secured by this mode of staining. It must be admitted that mordants are in some cases of use by enabling us to fix colouring matter in tissue elements that would otherwise be rebellious to staining. And they have in some cases the advantage of affording a very convenient means of regressive staining. For it happens that the colour-compounds thrown down in mordanted tissues are in many cases specially soluble in an excess of the mordant; so that the solution of the mordant itself forms a very appropriate decolourising agent.

---

* For an excellent popular exposition of this subject see BENEDIKT and KNECHT's 'Chemistry of the Coal-tar Colours' (George Bell and Sons).

Recognising these advantages, it must still, I think, be said that there seems to be some danger at the present moment that the practice of employing mordants may degenerate into an abuse. For surely the primary use and intention of an histological stain (not of an industrial dye) is, that it should select and reveal those elements of tissues that have a natural affinity for its colouring matter. That end is attained in the manner least open to objection by the use of substantive stains, the natural affinities of the tissues and the colouring matter here coming spontaneously and unconstrained into play. Not so in the case of adjective staining. Here the colour is as it were forcibly compelled into an unnatural union with all or many of the elements of the tissue, including many which have no natural affinity whatever for the colour. In such preparations the distinction between chromatic and achromatic elements is obliterated; and the interpretation of the images afforded by them is open to more serious causes of error than in the case of substantive stains.

Attention may be called here to the theoretically interesting "invert" stain obtained by Rawitz by the use of mordants (see *post,* Chap. XVI). In this curious process, a colour—safranin, for instance—which when used substantively is a pure chromatin stain, becomes by the use of mordants a pure plasma stain, staining cytoplasm deeply, and leaving the chromatin of nuclei absolutely unaffected. This strange effect can hardly be satisfactorily explained at present. Are we to suppose that the affinities of the nucleic acid of the chromatin are so fully satisfied by combination with the mordants, that it is unable further to combine with the colouring matter?

It may be noted in a general way that the following substances may be found useful as mordants, for enhancing the resistance of many tar colours to the alcohol employed for decolourising, and for producing a stronger stain.

Iodine: sections may be treated for a few minutes before staining with tincture of iodine.

Permanganate of potash : *see* HENNEGUY's process.

Formaldehyde : *see* OHLMACHER's process.

Aniline : *see* SAFRANIN.

Chromic acid : *see* BIZZOZERO's process.

215. Choice of a Stain.—The following may be recommended with confidence for general work :—*For sections,* MAYER's *hæmalum ;* or, for chromosmium objects more especially, *safranin* or *thionin.* If a plasma stain be required at the same time as a chromatin stain, I strongly recommend *Kernschwarz,* followed by *safranin* (see *Kernschwarz*).

For staining *in toto,* Grenacher's alcoholic *borax-carmine* or Mayer's *carmalum,* unless the object be so impermeable as to require a more highly alcoholised stain, in which case take Mayer's *paracarmine,* or for chromic acid objects Mayer's *hæmacalcium.*

For *fresh tissues* or small entire objects, *methyl green,* if it is not important to have permanent preparations ; if it is, take *carmalum* or *alum-carmine.*

Picric acid may be used for double-staining in bulk after carmine or hæmatoxylin.

Many others of the numerous stains discussed in the following chapters render most valuable services, and will be found recommended in the special paragraphs as occasion dictates.

The beginner will probably do well not to use a double stain where a single one will do. To do so is too often to go farther and fare worse.

216. Staining Reagents and Chemicals.—You are not likely to succeed in staining, especially in the beautiful processes of staining with coal-tar colours, unless you see to it that you are working with chemicals of the proper quality. You *cannot* ensure this by going to a generally trustworthy house for chemical products—at all events, not in the case of coal-tar colours. It is not sufficient that these should be what they are commercially described to be ; they may be pure, and yet not give good stains. They must (in the case of anilins, at all events) be the identical products used in their work by the authors who have described and recommended them (see the note on the numerous safranins in the market, *sub voce Safranin*). I therefore feel constrained to advise everybody to get his reagents—at all events his anilins—from the well-known chemists Grübler or Münder. Grübler has all the tried reagents in stock, and supplies only such as have been found by experiment with tissues to furnish the desired stain. He also makes up fixing and staining solutions, injection and imbedding masses, &c., according to the classical formulæ, and sends them out neatly packed and ready for use. From experience I can most highly recommend these preparations, which are in nine cases out of ten better than those the observer is likely to make for himself. They may be ordered from the price list, or by quoting the numbers of the formulæ in this work. The address is: Herrn Dr. G. Grübler & Co., Chemiker, Baiersche Strasse 63, Leipzig. Grübler can correspond in English.

His preparations can be obtained in London from Mr. R. Kanthack, 21, Golden Square, Regent Street, W., who is also the authorised agent for the microscopes and apparatus of Zeiss, also for the microtomes of Becker, and the bacteriological apparatus of F. & M. Lautenschlaeger, &c.

Münder's address is: Herrn Dr. G. Münder, Mikroskopisch-chemisches Institut, Göttingen.

Messrs. Squire & Sons, chemists, 413, Oxford Street, London, W., also make a speciality of microscopical reagents, and I am told furnish excellent products.

CHAPTER XIII.

CARMINE AND COCHINEAL STAINS.

217. The Theory of Carmine Staining.—I take the following from the important paper of MAYER, "Ueber das Färben mit Carmin, Cochenille, und Hämateïn-Thonerde," in *Mitth a. d. Zool. Station zu Neapel*, Bd. x, Heft 3, 1892, p. 480. The *rationale* of staining with carmine has hitherto been obscured by the erroneous notion that carmine is nothing but carminic acid with at most certain impurities. This is not the case. According to the analysis of LIEBERMANN (*Ber. d. Chem. Ges.*, Jahrg. 18, 1886, pp. 1969—1975) carmine is a *very peculiar alumina-lime-protein compound of carminic acid*, a true chemical compound from which at all events *aluminium* and *calcium* can no more be absent than sodium from salt. Analysis gives about 17 per cent. of water, 20 per cent. nitrogenous matters, 56 per cent. carminic acid, at least 3 per cent. alumina, and 3 per cent. lime, together with a small proportion of magnesia, potash, soda, phosphoric acid, and a trace of wax. Mayer has come to the conclusion that in the processes of histological staining (*not* of industrial dyeing) the active factors of the compound are, besides the carminic acid, only the alumina, and in some cases, the lime. *The other bases are inactive;* the nitrogenous matters, so far as they have any influence at all, are an obstacle, as it is they that give rise to the well-known putrefaction of the solutions.

Having arrived at these conclusions, it seemed logical to admit that *carminic acid, instead of carmine, should be taken as the basis of staining solutions.* This had already been proposed by DIMMOCK, whose paper (*Amer. Natural.*, xviii, 1884, pp. 324–7) I quoted at length in the first edition of this work. But Dimmock's proposals were not very successful, for the reason that he had omitted from his solutions the essential element, the alumina. He stained, for instance, with pure alcoholic solution of carminic acid, or of carminate of ammonia. Such solutions stain, but stain weakly and diffusely.

Mayer therefore sought for appropriate means of introducing the necessary alumina into the solutions; with the results that will be set forth in the next §.

218. Carminic Acid occurs as a purple-brown mass, easily soluble both in water and in alcohol. It is (according to Nietzki, *Chemie der organischen Farbstoffe,* Berlin, 1889, pp. 231—234) a weak (Liebermann says a strong) dibasic acid, which forms soluble salts with the alkaline metals, insoluble violet-coloured ones with the earthy and heavy metals. Very little is known concerning the chemical nature of these salts.

The alumina salt (carminate of alumina) has the remarkable property of being soluble not only in acids and acid salts, such as alum, but also in alkalies and alkaline salts, such as borax, provided that only water or weak alcohol be employed as the menstruum. It may be obtained by precipitating a solution of carminic acid or of carminate of ammonia by means of acetate of alumina. It is also precipitated from the above-named solutions by chloride of aluminium, but only in part; whilst if alum be taken no precipitate is produced, the carminate of alumina remaining in solution. Hence the composition of the staining fluid given below under the name of **Carmalum.**

When chloride of aluminium is taken, a precipitate of carminate of alumina is formed, as stated above. But this precipitate will redissolve if more chloride of aluminium be cautiously added. This gives the staining fluid described in § 224, which may be convenient in cases in which it is not desirable to work with a fluid containing alum.

Both of these solutions stain in a violet tone, something like alum-carmine. A redder tone may be obtained by adding calcium chloride to the carmalum solution. But this is not advisable, for calcium chloride added to carmalum precipitates the solution with formation of gypsum. Of course, this does not occur with the aluminium chloride solution; but for other reasons the addition does not give satisfactory results with the chloride of aluminium solution mentioned above. But it does give good results when combined with an *alcoholic* chloride of aluminium solution, and thus solves at once the problem of obtaining a *red* stain and an *alcoholic* staining fluid. This is described below under the name of **Paracarmine.**

If the foregoing explanations of the *rationale* of carmine staining be compared with the remarks on the theory of staining with hæmatoxylin given in the next Chapter, an interesting parallelism will be observed. In both processes, it is not the colouring matter alone which is active, but the colouring matter combined with *alumina.* The stain is always got with carminic acid + alumina, or with hæmateïn + alumina; other substances, such as lime, occasionally playing a part.

219. The foregoing considerations deal with the theory of staining with *Carmine;* we have now to consider the **Theory of Staining with Cochineal.** According to MAYER, whose earliest researches are confirmed by his latest (*Mitth. Zool. Stat. zu Neapel,* x, 3, 1892, p. 496), the active principle of extract or tincture of cochineal (as used in histology) is not free carminic acid, but carminic acid chemically combined with a base which is not lime, but some alkali. The pure aqueous extract contains only traces of lime, the alcoholic none at all. The watery extract made with *alum,* or cochineal-alum carmine (§ 226), owes its staining power to the formation of a carminate of alumina, the general properties of which have been discussed above when treating of the theory of carmine staining (§ 218). The tincture made with *pure alcohol,* on the other hand, contains only the above-mentioned carminate of some alkali. This carminate *alone* stains weakly and diffusely (like carminic acid alone). But if in the tissues treated with it it meet with lime salts, alumina or magnesia salts, or even metallic salts capable of combining with it and forming insoluble coloured precipitates in the tissues, then a strong and selective stain may result. As a matter of fact, the simple cochineal tincture of Mayer given in § 241 does give splendid results with certain objects (*i. e.* such as contain the salts in question). But it is unfortunately equally certain that such objects are rather rare than otherwise, and that with the majority of objects the stain is a very poor one.

But if the necessary salts be added to the tincture itself, then a solution ought to result containing the necessary elements for affording a strong and selective stain with all classes of objects. This proves to be the case; whence Mayer's new formula, § 242.

220. General Remarks.—What are the carmine stains useful for? Is it for staining fresh tissues? With the exception of aceto-carmine, no. Is it for staining sections? Again, no; for, in nine cases out of ten, sections are better stained by some of the anilin stains and by some hæmateïn stains than they can be in any carmine stain. Is it for staining entire objects?—for staining in the mass? Yes; for in many, if not in most cases, that can be done more satisfactorily by means of carmine than by means of any other known agent.

Hæmatoxylin (or hæmateïn) has a disastrous tendency to overstain; and the tar-colours are with hardly an exception entirely inapplicable to staining in bulk.

Overstains may in all cases be washed out with weak HCl (*e. g.* 0·1 per cent.). Henneguy (*Journ. de l'Anat. et de la Physiol.*, xxvii, 1891, p. 400) states that overstains may be completely removed by means of permanganate of potash. All carmine stains, with the exception of aceto-carmine, are permanent in balsam. None of the acid stains, nor any of Grenacher's fluids, should be used with calcareous structures that it is wished to preserve, unless they be taken in a state of extreme dilution.

221. Choice of a Carmine Stain.—Grenacher's alcoholic borax-carmine may be recommended to the beginner as being the easiest of these stains to work with.

In view of the far greater simplicity and precision of the methods proposed by Mayer, it is probable that they, or at all events methods conforming to the principles above laid down, will gradually take the place of the old methods. Not, indeed, that some of the old stains will not hold their ground. Alum-carmine will remain a superb stain, and borax-carmine remain superior to paracarmine in the power and brilliancy of its stain; but the superior precision of Mayer's methods will doubtless be acknowledged. And it is perhaps not too much to hope that fewer formulæ of the old roundabout, happy-go-lucky sort, with commercial carmine or hæmatoxylin as the chief ingredient, will henceforth be published.

In view of my conviction that the doctrines of MAYER concerning carmine staining are true, I have suppressed a very large number of formulæ that I hold to be as practically superfluous as they are theoretically irrational. See previous editions.

222. Pure Carminic Acid may be obtained from E. MERCK of Darmstadt, or from Dr. G. GRÜBLER & Co. (63, Bayersche Strasse, Leipzig). The price at present is from 2s. to 4s. per 10 grms.

Dr. MAYER writes me that the quality is sometimes not all that could be desired. Recent samples have been found to contain a perceptible quantity of ammonia or some fixed alkali.

A. AQUEOUS CARMINE STAINS.

a. Acid.

223. Mayer's Carmalum (*Mitth. Zool. Stat. zu Neapel*, x, 3, 1892, p. 489).—Carminic acid, 1 grm.; alum, 10 grms.; distilled water, 200 c.c. Dissolve with heat (if necessary: I have been able to make my solutions in the cold). Decant or filter. Add some antiseptic, either a few crystals of thymol, or 0·1 per cent. salicylic acid, or 0·5 per cent. salicylate of soda. The solution will then keep. A clearish red fluid with a violet tinge. It stains well in bulk even osmium objects. If washed out with distilled water only, the plasma will remain somewhat stained. If this be not desired, wash out carefully with alum solution, or, in difficult cases, with weak acid. The general effect is that of an alum-carmine stain. A notable difference between the two is that carmalum stains well in bulk, which alum-carmine is not very suitable for when used in the ordinary way; but see § 227.

A weaker solution may be made by taking from three to five times as much alum and five times as much water, and dissolving in the cold, which may be convenient. This is a very close equivalent of alum-carmine, giving, however, a somewhat redder stain. I find this solution very weak for ordinary work.

It should be noted that with either solution the objects to be stained should *not* have an *alkaline reaction.* The other properties of these solutions are very similar to those of alum-carmine.

224. **Mayer's Aqueous Aluminium-Chloride-Solution** (*Mitth. Zool. Stat. zu Neapel*, x, 3, 1892, p. 490).—Carminic acid 1 grm., chloride of aluminium, 3 grms.; water, 200 c.c. Add an antiseptic, as for carmalum.

Use as carmalum. The stain is of a blue-violet colour, very powerful, and elective. But it is not so pure a stain as that of carmalum, plasma being more strongly coloured. It is recommended only as a substitute for carmalum in cases in which the latter is counter-indicated on account of the presence of alum or the like.

225. **Alum-carmine** (Grenacher's formula, *Arch. mik. Anat.*, xvi, 1879, p. 465).—An aqueous solution (of 1 to 5 per cent. strength, or any other strength that may be preferred) of common or ammonia alum is boiled for ten or twenty minutes with ½ to 1 per cent. of powdered carmine. (It is perhaps the safer plan to take the alum solution highly concentrated in the first instance, and after boiling the carmine in it dilute to the desired strength.) When cool filter.

This stain must be avoided in the case of calcareous structures that it is wished to preserve.

Tizzoni (*Bull. Sc. Med. Bologna*, 1884, p. 259), Pisenti (*Gazz. degli Ospetali*, No. 24; *Zeit. f. wiss. Mik.*, ii, 1885, p. 378), and Grieb (*Mem. Soc. Ital. Sci.*, t. vi, No. 9, 1887; *Zeit. f. wiss. Mik.*, vii, 1, 1890, p. 47) have given modifications of Grenacher's formula which do not appear to me rational.

Alum-carmine is one of the best stains to be found outside the coal-tar colours. It is particularly to be recommended to the beginner, as it is easy to work with; it is hardly possible to overstain with it (except muscle). Its chief defect is that it is not very penetrating, and therefore quite unsuitable for staining objects of considerable size in the mass. This defect may, however, be to some extent overcome by employing the acid formula of Henneguy (§ 227), if it be not convenient to use Mayer's carmalum.

The stain is permanent in balsam, and in aqueous media if not acid.

226. **Cochineal Alum-Carmine** (Partsch, *Arch. f. mik. Anat.*, xiv, 1877, p. 180).—Powdered cochineal is boiled for some time in a 5 per cent. solution of alum, the decoction filtered, and a little salicylic acid added to preserve it from mould.

Another method of preparation has been given by Czokor (*Arch. f. mik. Anat.*, xviii, 1880, p. 413). Mayer has carefully examined both, and finds that Partsch's is the more rational, the proportion of alum in it being exactly right, whilst in Czokor's it is insufficient. Partsch's fluid has also the advantage of keeping better.

The formula known as Klein's cochineal fluid (which appears to have been first published in the *Ann. and Mag. Nat. Hist.*, viii, 1881, p. 232) is identical with that of Czokor.

The formula recommended by RABL in *Zeit. f. wiss. Mik.*, xi, 2, 1894, p. 168, is only an insignificant modification of that of Czokor.

Both these solutions are to all intents and purposes "alum-carmines." They give a stain that is practically identical with that of alum-carmine made from carmine, with perhaps even more delicate differentiations (but that depends so much on the quality of the carmine, the quality of the cochineal, and the nature of the objects to be stained that no absolute rule can be stated). On the whole, it seems to be a mere matter of convenience whether the one or the other should be preferred. The cochineal fluids should be used in exactly the same way as the carmine fluid.

It has been lately recommended by HERRICK (*Journ. Comp. Neur.*, Cincinnati, vol. i, 1891, p. 134) as a "vast improvement" to make the solution with sulphate of aluminium instead of alum (quoted from Mayer's paper, *Mitth. Zool. Stat. Neapel*, x, 3, 1892, p. 496).

227. Acetic Acid Alum-Carmine (HENNEGUY, *Traité des Méth. techn.*, LEE et HENNEGUY, 1887, p. 88).—Excess of carmine is boiled in saturated solution of potash alum. After cooling add 10 per cent. of glacial acetic acid, and leave to settle for some days. The deposit of carmine and alum that forms during that time is removed by filtration.

For staining, enough of the solution is added to distilled water to give it a deep rose tint. In order to ensure rapid diffusion, it is well to bring the tissues into the stain direct from alcohol. Stain for twenty-four to forty-eight hours, and wash for an hour or two in distilled water. (It is important that the water should be distilled in order to avoid the formation of crystals.) Dehydrate with alcohol and mount in balsam. You can mount in glycerin, but the preparations do not keep so well as in balsam.

The advantage of this carmine is that it has greater power of penetration than the non-acidified alum-carmine, and stains deep-seated layers of tissue just as well as the superficial ones. The colour of the stain is a somewhat inelegant violet, but this can be changed to a warmer tone by treating the objects with dilute HCl, as for borax-carmine objects.

228. Alum-Carmine and Picric Acid.—Alum-carmine objects may be double-stained with picric acid. LEGAL (*Morph. Jahrb.*, viii, p. 353) combines the two stains by mixing ten vols. of alum-carmine with one of saturated picric acid solution. I consider this to be a very recommendable practice.

229. Aceto-Carmine (Acetic Acid Carmine) (SCHNEIDER's formula, *Zool. Anzeig.*, No. 56, 1880, p. 254).—To boiling acetic acid of 45 per cent. strength add carmine until no more will dissolve, and filter. (Forty-five per cent. acetic acid is, according to Schneider, the strength that dissolves the largest proportion of carmine).

To use the solution you may either dilute it to 1 per cent. strength, and use the dilute solution for slow staining; or a drop of the concentrated solution may be added to a fresh preparation under the cover-glass. If you use the concentrated solution it *fixes* and stains at the same time, and hence may render service for the study of fresh objects. It is very penetrating, a quality that enables it to be used where ordinary reagents would totally fail. The stain is a pure nuclear one. Unfortunately the preparations cannot be preserved, and for this and other reasons the stain is *of very restricted applicability.*

ZACHARIAS (see *Zeit. f. wiss. Mik.*, v, 3, 1888, p. 371) adds to this solution wood vinegar (*acetum pyrolignosum*) in the proportion of 1 drop to 10 c.c.

A similar stain has been prepared with formic acid by PIANESE (see *Zeit. f. wiss. Mik.*, x, 4, 1894, p. 502). Probably for almost all the purposes for which aceto-carmine is useful, methyl-green will give better results.

230. Iron Carmine (ZACHARIAS, *Zool. Anz.*, No. 440, 1894, p. 62).—Stain thoroughly for several hours (Zacharias stains in an aceto-carmine, of which I suppress the formula, for, as pointed out to me by Dr. MAYER, and as I have verified, carmalum does just as well). Rinse the objects with dilute acetic acid, and bring them (taking care not to touch them with metallic instruments, if the aceto-carmine have been taken) into a 1 per cent. solution of ammoniated citrate of iron (the pharmaceutical *Ferri et Ammoniæ Citras*). Leave them till thoroughly penetrated, for as much as two or three hours if need be. In this solution they take on a black tint (with sections this happens in a few minutes). They should be removed as soon as the reaction has taken place throughout, otherwise there is risk of over-blackening. Wash for several hours in distilled water, dehydrate and mount in balsam.

This is at the same time a chromatin stain and a plasma stain. In my preparations chromatin is blue and plasmatic elements brown. I consider the method may render service in some cases. Amongst other things, spindles and spindle-relics are fairly well brought out by it. It can be applied to the coloration of entire objects (provided that they be small, MAYER).

β. *So-called "Neutral," and Alkaline.*

231. As to Picro-carmine.—The term "picro-carmine" is commonly used to denote a whole tribe of solutions in which carmine, ammonia, and picric acid exist *uncombined* in haphazard proportions. RANVIER, to whom we owe the invention of picro-carmine, claims that when prepared by his process it results as a definite chemical substance, a double salt of picric and carminic acid and ammonia, or *picro-carminate of ammonia.* But this has never been scientifically demonstrated. Picro-carmine gives good differentiations, and has the great merit of being less hurtful to most tissues than other aqueous alkaline carmines. It should be understood that the chief value of picrocarmine does not lie in its capacity of affording a double stain. The double stain, if that is all that is wanted, can be just as well or better obtained by staining first with borax-carmine, or the like, and after-staining with picric acid. The essential point about picro-carmine is that it is a fairly *neutral* fluid. Ranvier was, in fact, led to add picric acid to ammoniacal solution of carmine by the desire of neutralising the ammonia, that is all.

For slow staining, dilute solutions may advantageously have 1 or 2 per cent. of chloral hydrate added to them.

Overstains may be washed out with hydrochloric acid, say 0·5 per cent., in water, alcohol, or glycerin.

Preparations should be mounted in balsam, or if in glycerin this should be acidulated with 1 per cent. of acetic or, better, formic acid.

232. RANVIER'S **Picro-carmine or "Picro-carminate of Ammonia."**—The method of preparation employed in the Laboratory of Histology of the Collège de France, kindly communicated to myself and Henneguy for our *Traité des Méth. techn.* (q. v. p. 451) by M. VIGNAL, one of the assistants there, is as follows:

Take—

Water	1000 parts.
Picric acid	20 ,,
Carmine	10 ,,
Ammonia	50 ,,

Put them into a stoppered bottle and leave them for two or three months in a warm place. Then put them into a large crystallising dish and let them putrefy. When the liquid has become reduced by evaporation to four fifths of its original volume, remove the crystals that have formed at the bottom, dry them, and dissolve them in a little warm water. Filter the solution, and examine it with the microscope to see whether the carmine is really dissolved. If not, add water and ammonia, and let the solution putrefy again; evaporate and examine as before. When you have got your carmine combined, evaporate the solution to dryness in a stove, and reduce the picro-carminate to powder.

For staining, dissolve 1 grm. of the powder in 100 grms. of water, and add a crystal of thymol to prevent the development of mould.

233. Ranvier's Original Formula (*Traité*, p. 100) was as follows:—To a saturated solution of picric acid add carmine (dissolved in ammonia) to saturation. Evaporate down to one fifth the original volume in a drying oven; and separate by filtration the precipitate, pour in carmine, that forms in the liquid when cool. Evaporate the mother-liquid to dryness, and you will obtain the picro-carminate in the form of a crystalline powder of the colour of red ochre. It ought to dissolve completely in distilled water; a 1 per cent. solution is best for use.

234. Other Formulæ for Picro-carmine.—I have tried most of the following, and found no real advantage in any of them:—Gage, *Am. M. Mic. Journ.*, i, 1880, p. 22; *Journ. Roy. Mic. Soc.*, vol. iii, p. 501 (very elaborate and has not afforded me a soluble carmine.). Fol, *Lehrb. d. vergl. mik. Anat.*, p. 195. Rutherford, *Pract. Hist.*, p. 173. Paul Mayer, *Mitth. Zool. Stat. Neapel*, ii, p. 20. Baber, *Mon. Micro. Journ.*, xii, p. 48. Pergens, Carnoy's *Biologie cellulaire*, p. 92. Hoyer, *Biol. Centralb.*, ii, 1882, p. 17. Bizzozero, *Zeit. f. wiss. Mik.*, 1885, p. 539. Klemensiewics, *Sitzb. Akad. Wiss. Wien*, lxxviii, 1878, iii, Juni; *Zeit. f. wiss. Mik.*, i, 1884, p. 501. Cuccati, *Zeit. f. wiss. Mik.*, vi, 1, 1889, p. 42.

For *Soda-Picro-Carmine* see Löwenthal, *Anat. Anzeig.*, ii, 1887, No. 1, p. 22, and *Zeit. f. wiss. Mik.*, x, 3, 1893, p. 313; Anon., *Journ. Roy. Mic. Soc.*, 1888, p. 518; Squire's *Methods and Formulæ*, &c, 1892, p. 35.

Weigert corrects unsatisfactory samples of picro-carmine in the following way (*Virchow's Archiv*, Bd. lxxxiv, pp. 275, 315; *Zool. Jahrb.*, 1881, p. 40):—Small quantities of acetic acid are added "until the first slight precipitate appears even after stirring." The whole is again put away for twenty-four hours more, when it will be found that there has formed a precipitate that can only partially be removed by filtration; ammonia is then added drop by drop at intervals of twenty-four hours, until the solution becomes clear. If the solution stains too yellow, acetic acid is added; if it overstains red, a little ammonia is again added. All badly staining samples of picro-carmine may, according to Weigert, be improved in the same way by addition of acetic acid or ammonia. I consider it to be a very happy-go-lucky process.

235. Lithium-Carmine (ORTH, *Berlin. klin. Wochenschr.*, xxviii, 1883, p. 421).—Superfluous, and macerates considerably (MAYER).

236. Ammonia-Carmine.—In my opinion there is no valid excuse for using ammonia-carmine at all at the present day.

If, however, such a stain be used, care should be taken to get rid of the free ammonia as completely as possible. This may be done by boiling until the excess of ammonia has evaporated. (So long as free ammonia is present *large* bubbles are formed in the fluid, and the latter shows a dark purple colour. When the free ammonia has evaporated *small* bubbles appear, and the solution takes a brighter red tint.)

One per cent. each of carmine and ammonia in distilled water is a good proportion.

But a safer mode of preparation is that of RANVIER, as follows (kindly communicated by Dr. MALASSEZ, see *Traité des Méthodes techniques*, &c., of Lee and Henneguy, 1st. edit., p. 82).—Make a simple solution of carmine in water with a *slight* excess of ammonia, and expose it to the air in a deep crystallising dish until it is entirely dried up. It should be allowed to putrefy if possible. Dissolve the dry deposit in pure water, and filter.

γ. *Other Aqueous Carmines (Acid and Alkaline).*

237. SCHWEIGGER-SEIDEL'S **Acid Carmine** (RANVIER, *Traité*, p. 99). HAMANN'S **Acid Carmine** (*Intern. Mon. f. Anat. u. Hist.*, i, 5, 1884; *Zeit. f. wiss. Mik.*, ii, 1885, p. 87). **Neutral Borax-Carmine** (NIKIFOROW, *Zeit. f. wiss. Mik.*, v, 3, 1888, p. 337). **Neutral Borax-Carmine** (GRENACHER, *Arch. f. mik. Anat.*, xvi, 1879, p. 466). HAUG'S modifications of this, *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 151, and viii, 1, 1891, p. 52. WOODWARD'S **Borax-Carmine** (see *Monthly Mic. Journ.*, vii, 1872, p. 38; *Am. Quart. Mic. Journ.*, i, 1879, p. 220; *Journ. Roy. Mic. Soc.*, ii, p. 613). H. GIBBES' **Borax Carmine** (see *Journ. Roy. Mic. Soc.*, iii, 1883, p. 390). DELAGE'S **Osmium-Carmine** (*Arch. de Zool. exp. et gén.*, iv, sér. 2, 1886; *Zeit. f. wiss. Mik.*, iii, 2, 1886, p. 240). ROLLETT'S **Carminroth** (see *Zeit. f. wiss. Mik.*, i, p. 91). PERL'S **Soluble Carmine** (see FREY, *Das Mikroskop.*, 7 *Auf.*, and *Zeit. f. wiss. Mik.*, i, p. 91). **Carminic Acid** (see DIMMOCK, *Amer. Natural.*, xviii, 1884, pp. 324–7; and *Journ. Roy. Mic. Soc.*, 1884, pp. 471–4). **Boric Acid Carmine** (ARCANGELI, see *Proc.-verb. Soc. Toscana Sc. Nat.*, 1885, p. 283; and *Zeit. f. wiss. Mik.*, 1885, p. 377). **Boric Acid Alum-Carmine,** ARCANGELI, *ibid.* **Salicylic Acid Alum-Carmine,** ARCANGELI, *ibid.* **Salicylic Acid Carmine,** ARCANGELI, *ibid.* **Picric Acid Carmine,** ARCANGELI, *ibid.* **Picric Acid Carmine,** MINOT (see WHITMAN'S *Methods in Mic. Anat.*, p. 42). **Uranium-Carmine,** GIERKE, *Zeit. f. wiss. Mik.*, i, 1884, p. 92; SCHMAUS, *ibid.*, viii, 2, 1891; cf. *Münchener med. Wochenschr.*, 1891, No. 8, p. 147. **Carbonate of Soda Carmine,** CUCCATI, *Zeit. f. wiss. Mik.*, iv, 1, 1887, p. 50.

B. ALCOHOLIC CARMINE STAINS.

238. Alcoholic Borax-Carmine (GRENACHER, *Arch. f. mik. Anat.*, xvi, 1879, p. 466, *et seq.*).—Take a *concentrated* solution of carmine in borax solution (2 to 3 per cent. carmine to 4 per cent. borax); boil it for half an hour or more; dilute it with about an equal volume of 70 per cent. alcohol, allow it to stand some time (twenty-four hours—MAYER), and filter. Or the mixture of carmine and borax solution is *allowed to stand for two or three days* and occasionally stirred; the greater part of the carmine will dissolve. To the solution is added an equal bulk of 70 per cent. alcohol; the mixture is allowed to stand for a week, and then is filtered. If on keeping more carmine is deposited, it must be refiltered.

Preparations should remain in the stain until they are thoroughly penetrated (for days if necessary), and then be brought (*without first washing out*) into alcohol (of 70 per cent.; this is absolutely necessary—MAYER) acidulated with 4 to 6 drops of hydrochloric acid to each 100 c.c. of alcohol. They are left in this until the stain is differentiated, and may then be washed or hardened in neutral alcohol. Four drops of HCl is generally enough. Three drops I find not quite sufficient. The stained objects should remain in the acidulated alcohol till they acquire a bright transparent look. This may require days (MAYER).

For delicate objects, and for very impermeable objects, it may be well to increase the proportion of alcohol in the stain; it may conveniently be raised to about 50 per cent. It should not exceed 60 per cent. in any case (MAYER).

This stain is probably by far the most popular of any for staining in the mass. It is easy to use, and gives a most splendid coloration. But it is not so penetrating as is commonly supposed, and has the defect of sometimes forming precipitates in the cavities of bulky objects which cannot be removed by washing out. And it must be remembered that the fluid is alkaline, and therefore not suitable for delicate cytological work. I believe that some otherwise excellent cytological work has been vitiated by over-confidence in this reagent, and will have to be done over again.

239. MAYER'S **Paracarmine** (*Mitth. Zool. Stat. zu Neapel*, x, 3, 1892, p. 491).—Carminic acid, 1 grm.; chloride of aluminium, 0·5 grm.; chloride of calcium, 4 grms.; 70 per cent. alcohol, 100 c.c. Dissolve cold or warm, allow to settle, and filter. A light red liquid, specially adapted for staining in bulk, and the nearest approach to a substitute for Grenacher's alcoholic borax-carmine that has yet been discovered.

Objects to be stained *should not have an alkaline reaction*, nor contain any considerable amount of carbonate of lime (spicules or skeletal parts of corals, &c.), which would give rise to precipitates (MAYER). Wash out sections, or objects intended to be sectioned, with pure 70 per cent. alcohol. Objects intended to be mounted whole may be washed out with a weak solution of aluminium chloride in alcohol, or if this be not sufficient, with 5 per cent. common acetic acid (or 2·5 per cent. glacial acetic acid) in alcohol.

For staining bulky objects with large cavities, such as *Salpa*, the solution should be diluted (with alcohol); and as this may cause precipitates to form during the staining, especially if the objects are not very clean, it is advisable to *slightly acidify the dilute solutions*.

Paracarmine gives a nuclear stain of a red colour, though not so fiery red as that of borax-carmine. Its points of superiority over borax-carmine are that it is not alkaline, therefore less hurtful to tissues; that it is more highly alcoholic, therefore more penetrating; that it has less tendency to form granular precipitates in the interior of objects, and that it generally keeps perfectly without precipitating (mine has precipitated somewhat, though not to an injurious extent).

240. Alcoholic Hydrochloric-Acid Carmine.—In view of the abovementioned defects of borax-carmine—defective penetration, and a tendency to form insoluble granular precipitates in the interior of objects—it is desirable to possess a powerful staining medium more highly alcoholised and of acid reaction. Hydrochloric acid carmine possesses these qualities, and may be useful in the case of objects for which Mayer's paracarmine may not suffice. It may, for instance, be frequently useful in work on Arthropoda, especially the marine forms.

GRENACHER's receipt (*Arch. f. mik. Anat.*, xvi, 1879, p. 468) will be found extremely troublesome by those who are not expert at neutralising. The following method, due to PAUL MAYER (quoted from GARBINI's *Manuale per la Technica moderna del Microscopio*, first ed., p. 46), is easy and gives excellent results. Take 100 grms. of alcohol (either absolute or of any weaker grade), 1 or 2 drops of HCl, and *an excess* of carmine, and boil until you get a clear solution, taking care that there remains *an excess of carmine*. This ought to give a nuclear stain, without the aid of HCl for washing out. If overstaining or diffusion should occur, wash out with alcohol, *very slightly acidulated* with HCl.

Brass (*Zeit. f. wiss. Mik.*, ii, 1885, p. 303) takes 100 c.c. of 70 per cent. alcohol, 15 drops of HCl, and an excess of carmine. An old formula of Paul Mayer's (*Mitth. Zool. Stat. Neapel*, iv, 1883, p. 521; *Journ. Roy. Mic. Soc.* [N.S.], iv, 1884, p. 317), which gives a more powerful stain than the preceding, is somewhat inconvenient to carry out. A more recent formula of Mayer's (*Intern. Monatsschr. f. Anat., &c.*, 1887, p. 43) is as follows: Carmine, 4 grms.; water, 15 c.c.; hydrochloric acid, 30 drops. Boil till the carmine is dissolved, add 95 c.c. of 85 per cent. alcohol, and neutralise by adding ammonia until the carmine begins to precipitate.

If it be desired to dilute any of these solutions, it should be done with alcohol, not water, and alcohol should be taken for washing out.

241. Alcoholic Cochineal (Mayer's **Old Formula**, *Mitth. Zool. Stat. Neapel*, ii, 1881, p. 14).—Cochineal in coarse powder is macerated for several days in alcohol of 70 per cent. For each gramme of the cochineal there is required 8 to 10 c.c. of the alcohol. Stir frequently. Filter, and the resulting clear, deep red solution is fit for staining.

The objects to be stained must previously be imbibed with alcohol of 70 per cent. and alcohol of the same strength must be used for washing out or for diluting the staining solution. The washing out must be repeated with fresh alcohol until the latter takes up no more colour. Warm alcohol acts more rapidly than cold. Overstaining seldom happens; it may be corrected by means of 70 per cent. alcohol, containing $\frac{1}{10}$ per cent. hydrochloric or 1 per cent. acetic acid.

Small objects and thin sections may be stained in a few minutes, larger animals require hours or days.

A nuclear stain, slightly affecting protoplasm. The colour varies with the reaction of the tissues, and the presence or absence of *certain salts* in them. Crustacea with thick chitinous integuments are generally stained red, most other organisms blue. The stain is also often of different colours in different tissue elements of the same preparation. Glands or their secretion often stain grey-green. In embryos of *Lumbricus* Kleinenberg found the vessels to stain red, their contents of an intense blue.

Acids lighten the stain and make it yellowish red. Caustic alkalies turn it to a deep purple.

The best stains are obtained in the case of objects that have been prepared with chromic or picric acid combinations, or with absolute alcohol. Osmic acid preparations stain very weakly unless they have been previously *bleached.* The acid must be carefully washed out before staining, or a diffuse stain will result. The stain is permanent in oil of cloves and balsam.

The object for which this stain was imagined is to obtain an *alcoholic* staining fluid whose high penetrating power allows it to be employed in the case of organisms, such as Arthropoda, whose chitinous investments are but very slightly permeable by aqueous solutions of carmine.

I have treated this stain at considerable length because I am convinced that it ought to be better known. It is very useful in many cases (Annelids, for instance), and indispensable for Arthropoda. On the other hand, it gives a very poor stain with unsuitable objects, and the majority of the objects with which the histologist has to do are in this case. For this reason MAYER has been led to devise the fluid described in the following paragraph, which, containing in itself the salts necessary for producing a rich and energetic selectivity of stain, gives good results with *all classes of objects.* He almost appears to intend it entirely to take the place of the old fluid. But I think the old fluid cannot yet be discarded. It has over the new fluid the (for some cases considerable) advantage of being more highly alcoholic; and it does not contain free acid, so that it *can be used with calcareous structures* which it is wished to preserve—which the new fluid cannot. For specimens of *Pluteus,* for instance, I find it excellent.

242. MAYER'S **Alcoholic Cochineal, New Formula** (*Mitth. Zool. Stat. Neapel,* x, 3, 1892, p. 498).—Cochineal, 5 grms.; chloride of calcium, 5 grms.; chloride of aluminium, 0·5 grm.; nitric acid of 1·20 sp. gr., 8 drops; 50 per cent. alcohol, 100 c.c. Powder the cochineal finely and rub up in a mortar with the salts, add the alcohol and acid, heat to boiling-point, leave to cool, leave for some days standing with frequent agitation, filter.

Use as the old tincture, the objects being prepared and washed out with 50 per cent. alcohol. The stain is like that of paracarmine, but not quite so strong and not so sharp. Mayer only recommends it as a *succedaneum* of paracarmine.

The object for which it was devised has been explained in the last paragraph, where also some limitations to its usefulness have been indicated.

For the chemical theory involved in the composition of these tinctures see also the last paragraph, and *supra,* § 219.

CHAPTER XIV.

HÆMATEIN (HÆMATOXYLIN) STAINS.

243. Theory of Hæmatoxylin Staining.—It appears to be now thoroughly well established (see Nietzki, *Chemie der organischen Färbstoffe,* Berlin, Springer, 1889, pp. 215—217) that the active colouring principle of hæmatoxylin dyes is *hæmatein ;* and further, that the hæmatein of the usual histological staining solutions is a product of the oxidation of their contained hæmatoxylin by means of the air to which they are exposed (see Mayer, "*Ueber das Farben mit Hämatoxylin,*" in *Mitth. a. d. Zool. Station zu Neapel,* Bd. x, Heft 1, 1891, pp. 170—186; Unna, "*Ueber die Reifung unserer Färbstoffe,*" in *Zeit. f. wiss. Mik.,* viii, 4, 1892, p. 483). This change is known as "ripening," and until it has taken place the solutions are not fit to use for staining.

Hitherto it has been the practice to rely (quite unconsciously so far as the chemical theory is concerned) on the spontaneous absorption by the solutions of oxygen from the air to effect this "ripening," but it has now been discovered (by both Mayer and Unna independently) that nothing is easier than to bring about the reaction artificially; all that is necessary being, for instance, to add to a solution of hæmatoxylin containing alum a little neutralised solution of peroxide of hydrogen. The solution becomes almost instantaneously dark blue, "ripe," and fit for staining, thus definitely confirming the truth of the hypothesis.

Mayer goes further. A solution of pure uncombined hæmatein would not afford a selective stain such as we require in histology; it would be at most a dye. The usual solutions (I am not here speaking of Weigert's or similar processes) all contain alum, and Mayer holds that the active agent in them is a compound of hæmatein with alumina ("Hæmateïn-Thonerde"—hæmateate of alumina). He holds that this salt is precipitated in the tissues (chiefly in the nuclei) by certain organic and inorganic salts there present (for instance, phosphates); perhaps also by other organic bodies belonging to the tissues. Such salts are known to exist in living tissues. By the process of "fixing" they are fixed there,—either in the same chemical state, as when such an agent as alcohol is used for fixing; or as fresh compounds formed by the action of the fixing agent itself, as when such a fixing medium as sublimate is used.

The chief factor in the composition of these stains is, therefore—after the colouring substance itself,—*alumina,* to which, however, may be added some other bodies, such as lime, which may play a secondary *rôle* (as, for instance, chloride of calcium does in Kleinenberg's solution).

It follows that the first difficulty with which the worker with hæmatoxylin stains has to contend is that of getting his hæmatoxylin duly oxidised into hæmatein, in order to the formation of the desired hæmatein-alumina compound, or lake. If this be done by the hitherto customary process of leaving the solutions to "ripen" by the action of the air, it is necessary to wait for a long time before the reaction is obtained. During all this time, it may be weeks or months, there is no means, except repeated trial, of ascertaining whether the solution at any moment contains sufficient hæmatein to afford a good stain. And here a second difficulty arises; the oxidising process continuing, the solutions become "over-ripe;" the hæmatein, through further oxidation, passes over into colourless compounds, and the solutions begin to precipitate. They are therefore, in reality, a mixture in constantly varying proportions of "unripe," "ripe," and "over-ripe" constituents (the first and last being useless for staining purposes), and, in consequence, their staining power is very inconstant.

These difficulties are only very imperfectly got over by any of the very various methods that have been proposed until lately. Now, however, thanks to the important discoveries of MAYER and UNNA, a great step in advance has been taken.

The great point in Mayer's work is that not hæmatoxylin, but *hæmatein,* should be taken in the first instance for making the staining solutions. This at once relieves us from the tedious and uncertain process of "ripening" in the old way. We have a ripe solution to begin with, and we know that it must be ripe. A discovery of Unna's, to be mentioned below, affords a means of preventing the "over-ripening" brought about by excessive oxidation.

244. Unna's Half-ripe Constant Stock Solution (*Zeit. f. wiss. Mik.*, viii, 4, 1892, p. 483).

This result may be attained by simply adding a *reducing agent* to the solution. Various reducing agents are available for this purpose (see the original paper); the most convenient method is the simple addition of a little sulphur. The following formula is recommended:

Hæmatoxylin	1
Alum	10
Alcohol	100
Water	200
Sublimed sulphur	2

If the sulphur be added to the hæmatoxylin solution only when the latter has become somewhat strongly blue, *i. e.* after two or three days' time, the stage of oxidation attained by the solution will be fixed by the sulphur. The solution in this state may be used for staining. It will not give so energetic a stain as the solutions totally ripened with peroxide of hydrogen (see the paper quoted), and the stain will be somewhat more diffuse, but washing out with acids will not be necessary. If an energetic and more purely nuclear stain be desired, the solution should be treated with neutralised peroxide of hydrogen, as described in the paper quoted; but this process is cumbrous and unnecessary, and I suppress it. Mayer (*Mitth. Zool. Stat. Neapel*, xii, 2, 1896, p. 309) finds that the sulphur process does not preserve the solutions for long, whilst for some unexplained reason the simple addition of *glycerin* does; see below, "Glychæmalum."

245. Concerning Hæmatein.—The following is taken from the two papers of Mayer quoted above, §§ 217, 243. Hæmatein occurs as a dark green mass, red by transmitted light, which may be rubbed up into a violet powder. It is entirely, though with difficulty, soluble in distilled water and in alcohol, giving a yellowish-brown solution, which remains clear on addition of acetic acid. Alkalies dissolve it with a blue-violet tint.

It is now found in commerce; but Mayer has hitherto only been able to procure it in a pure state from Geigy and Co., in Bâle. But there is also found in commerce an ammonia-compound of hæmatein—*Hæmateïn-Ammoniak,* also known in commerce as *Hæmateinum crystallisatum;* this may be obtained in a sufficiently pure state from E. Merck, of Darmstadt.

This is somewhat more easily soluble in both water and alcohol than hæmatein is, and does quite as well for staining purposes. The histologist can easily prepare it for himself as follows:

246. Hæmateate of Ammonia (Mayer, *ibid.*).—Dissolve 1 grm. of hæmatoxylin with the aid of heat in 20 c.c. of distilled water, filter if necessary, add 1 c.c. of caustic ammonia (of 0·875 sp. gr.), and bring the purple liquid into a capsule of such dimensions that its bottom be not covered to a depth of more than half a centimetre. Let the liquid evaporate at the ordinary temperature and protect it from dust. The dry product will consist of hæmateate of ammonia, about equal in weight to the hæmatoxylin taken in the first instance. The evaporation should not be hastened by heat, as this may give rise to the formation of substances that are insoluble in alcohol. The preparation should not be touched, until it is dry, with any other instruments than such as are made of glass, porcelain, or platinum.

247. Characters of Hæmatein Stains (or of Hæmatoxylin Stains).—It is evident, from what has gone before, that all so-called hæmatoxylin stains are really produced by means of hæmatein. The alum-hæmatein lakes stain in **different tones of blue or of red**, according to the composition of the staining solution. Neutral or alkaline solutions give a blue stain; acid solutions give a red one. In order to *get a blue stain* in preparations that have come out red through the acidity of the staining bath, it is a common practice to treat them with weak ammonia, in the belief that the blue colour is restored by neutralisation of the acid that is the cause of the redness. According to Mayer, the ammonia acts not by neutralising the acid, but by precipitating the alumina, which carries down the hæmatein with it (if no alumina were present the colour would be purple, not blue). The *same result* can generally be obtained by merely washing out with common tap-water, which is usually sufficiently alkaline (Squire has obtained the reaction with distilled water, free from even a trace of ammonia), and can be obtained with certainty by treatment with bicarbonate of soda or acetate of soda or potash. And this is the preferable course, as ammonia is certainly a dangerous thing to treat delicate tissues with. Of course this is a different question from that of *neutralising* with an alkali tissues that have been treated with an acid to correct over-staining. Here the neutralisation may be indicated in the interest of the *preservation* of the stain, or of the tissues themselves.

Squire (*Methods*, p. 22) finds that sections can be blued in a few seconds by treatment with a 1 : 1000 solution of bicarbonate of soda in distilled water. Mayer holds that acetate of potash is the most inoffensive reagent to take; a strength of 0·5 to 1 per cent. may be taken.

According to Watney (see *Phil. Trans.*, 1812, p. 1085; Krause, *Intern. Zeit. f. Anat. u. Hist.*, i, p. 154; and M. Flesch, *Zeit. f. wiss. Mik.*, 1885, p. 358, from whom I quote) the colour is an intense blue if the solution has been made *with freshly prepared alum*, whilst a red tone is obtained if the solution has been made with old alum. The reason of this is that alum that has been long kept almost always contains free acid. The red solutions are said to exhibit a great affinity for connective tissue, and for the granules of "plasma-cells;" whilst the blue solutions show a special affinity for mucin and chromatin. But see on this point the remarks of Mayer quoted under "**Mucin.**"

It has been discovered by Langhans (see Max Flesch, *loc. cit.*) that it is possible to obtain these two elective reactions with one and the same solution. All that is necessary is to stain with the solution of Delafield, mount the preparations in balsam, and expose them for some time to the light. The reaction is not obtained with glycerin mounts.

None of the purely aqueous solutions are perfectly stable; only one or two are fairly so. They can, however, be made to keep for a sufficient time, many months, by the addition of glycerin (Ehrlich); but the addition of the glycerin, as is well known, slows the stain greatly, and makes it somewhat less precise. (See below, *Glychæmalum*).

It has been explained above that the old hæmatoxylin solutions when freshly prepared stain badly and diffusely; they ought either to be "ripened" by Unna's method (§ 243) or to be allowed to "ripen" spontaneously before use. This takes, according to the nature of the solution, a few hours, or days, or months. In my opinion there is no legitimate excuse for not making up stains with hæmatein, and the old formulæ for hæmatoxylin solutions ought to be discarded.

All of the solutions, when in good staining order, have a great tendency to over-stain. Over-stains may be corrected by washing out with weak acids (*e. g.* 0·1 to 0·2 or even 0·5 per cent. of hydrochloric acid), but this is not favorable to the permanence of the stain. If acids be used, it is well to re-neutralise afterwards with ammonia or bicarbonate of soda (0·1 per cent.).

Bicarbonate of soda may be used for neutralisation with alcohol as the vehicle. It should be dissolved by the aid of heat in 70 per cent. alcohol (very little will dissolve), and the solution filtered after cooling. For neutralising tissues, three to five drops of the solution are added to a watch-glass full of 70 per cent. alcohol in which the tissues are soaked (VON WISTINGHAUSEN, *Mitth. Zool. Stat. Neapel,* x, 1891, p. 41; *Zeit. f. wiss. Mik.,* x, 4, 1893, p. 480).

The best plan of all is to avoid over-staining by staining very carefully in extremely dilute solutions. RAWITZ (*Anat. Anz.,* xi, 10, 1895, p. 301) gives the proportion of one to three drops of a strong alum-hæmatein solution to 25 to 50 c.c. of distilled water. Sections of picric or sublimate material may remain in this without over-staining for twenty-four to forty-eight hours. Chrom-osmium material is of course much less likely to over-stain, but even in this case the best results are obtained with very dilute solutions. It should be noted that the purest chromatin stains are obtained by staining for a short time (sublimate sections half an hour, say) in solutions of *medium* strength, such as hæmalum diluted ten to twenty-fold with water. The stain obtained either with very strong solutions, or with the slow stain of the dilute solutions, is at the same time a plasma-stain, which of course may or may not be desired. Chrom-osmium material will not yield a pure chromatin stain unless it is very *fresh;* it is consequently next to impossible to obtain the reaction with paraffin sections of such material; they constantly give a plasma-stain in addition to the chromatin stain, which is not the case with sublimate material. Chrom-osmium material, if not quite fresh, will require at least as many hours' staining as sublimate material does minutes.

The stain is fairly permanent in balsam, but is sure to fade a little, and may fade a great deal. If acids have been used after staining, great care should be taken to wash them out thoroughly before mounting. In aqueous media the stain cannot be relied on to keep (this refers to the old solutions: MAYER finds that his hæmatein preparations have kept well both in aqueous media and, with certain precautions, in balsam). Turpentine-balsam should not be used (Mayer, *in litt.*), nor, I find, should alcohol-balsam.

248. General Remarks.—We have the coal-tar colours for staining sections, and we have carmine and cochineal for staining in the mass. What, then, do we want hæmatein for? The answer is that we sometimes want it for staining, either sections or in the mass, on account of the faculty it has of staining tissues that have been treated with chromic and osmic mixtures. This it does in general better than any carmine or cochineal. We want it also for some special purposes, such as staining the *Nebenkern* and achromatic figures of nuclei, and for nerve researches and other special histological objects. It is also a more powerful stain than carmine; and according to the mode of employment affords either a chromatin stain or a sometimes valuable plasma-stain. The chief hæmatein plasma-stains are found not amongst the alum-hæmatein lakes, but amongst the other lakes, iron or chrome lakes, &c. The alum lakes will be treated of first; then the other lakes, partly in this chapter and partly in Part II (Neurological Methods, Methods of WEIGERT, PAL, &c.).

In view of the very great advantages offered by the mode of preparation worked out by MAYER I have no hesitation in saying that the old methods for preparing alumina-hæmatein lakes ought to be entirely discarded, and Mayer's formulæ only, or at least only such formulæ as conform to the principles laid down by him, used instead. For not only are his modes of preparation the most scientific, but, according to my experience, his solutions are found in practice to be greatly superior to the old ones.

A. *Alumina-hæmatein Lakes.*

249. MAYER'S **Hæmalum (Alum-hæmatein Solution, Hæmalaun**; MAYER, *Mitth. a. d. Zool. Stat. zu Neapel*, x, 1, 1891, p. 172; see also *Zeit. f. wiss. Mik.*, viii, 3, 1891, p. 338, a very exact report, but somewhat short considering the importance of Mayer's paper).—One grm. of the colouring matter (either hæmatein or the ammonia salt, §§ 245, 246) dissolved with heat in 50 c.c. of 90 per cent. alcohol, and added to a solution of 50 gr. of alum in a litre of distilled water. Allow the mixture to cool and settle, filter if necessary, and add a crystal of thymol to preserve from mould.

A dark liquid of about the tint of Grenacher's borax-carmine. It answers to Böhmer's hæmatoxylin,* stains at least as well, *either at first, for it is ripe from the beginning, or later.* It may be used either in the concentrated form or diluted. Concentrated, it stains almost instantaneously (sections have been stained by merely pouring the liquid over them). Diluted twentyfold with distilled water it will still stain through the tentacles of a *Tubularia* in an hour. (Spring water or tap water containing lime must not be used for diluting; perhaps weak solution of alum in distilled water is the best means of all.) After staining, sections may be washed out either with distilled or common water. The solution is *admirable for staining in bulk.* Large objects will, however, require twenty-four hours' staining, and should be washed out for the same time (this should be done with 1 per cent. alum solution if a sharp nuclear stain be desired). The solution unfortunately does not keep perfectly, but becomes weak with age (see § 251). Mayer's preparations have kept well in glycerin (care being taken not to have it acid), also in balsam. It is to be noted that if oil of bergamot be used for clearing, it must be thoroughly removed by means of oil of turpentine before mounting; and that oil of cloves is dangerous. It is best (Mayer, *in litt.*) to use only xylol, benzol, or chloroform, and to mount in xylol-balsam or chloroform-balsam.

Hæmalum may be mixed with alum-carmine, Säurefuchsin, or the like, to make a double-staining mixture; but it seems preferable to use the solutions in succession.

250. Mayer's **Acid Hæmalum** (*ibid.*, p. 174, note).—This is hæmalum with 2 per cent. glacial acetic acid (or 4 per cent. common acetic acid). To be used as the last, washing out with ordinary water in order to obtain a blue-violet tint of stain. It has the advantage of being a perhaps even more precise nuclear stain than the last formula; and further, the solution does not precipitate through decomposition caused by the ammonia of the air and the alkali of the glass of bottles, which the simple hæmalum solution does to a certain extent (though this does not affect its staining power).

---

* As with Böhmer's formula, it is not necessary to conform exactly to the proportions given, and a rough and ready hæmalum solution may be at any time extemporised by adding a few drops of alcoholic solution of hæmatein to an alum solution of any desired strength.

251. Mayer's **Glychæmalum** (*Mitth. Zool. Stat. Neapel*, xii, 2, 1896, p. 310).—Mayer has found that hæmalum does not keep perfectly (I have also found that my solution has not kept in a sufficiently satisfactory manner). By adding glycerin it may be made to keep perfectly clear for over a year; but as this slows the stain, more hæmatein should be added. The following formula is recommended :—Hæmatein 0·4 grm. (to be rubbed up in a few drops of glycerin in a mortar till it dissolves); alum, 5 grms.; glycerin, 30; distilled water, 70. The stain is not quite so purely nuclear as that of the simple hæmalum,—ground substance of cartilage, for instance, being stained.

252. Hansen's **Solution** (*Zool. Anz.*, 1895, p. 158).—Hansen having found that commercial hæmatein is not of constant quality, sought for a convenient means of oxidising hæmatoxylin into hæmatein,and recommends the following process.

One grm. of hæmatoxylin crystals is dissolved in 10 grms. of absolute alcohol. Twenty grammes of potash alum are dissolved with the aid of heat in 200 grms. of distilled water, and the solution filtered after cooling. After twenty-four hours the two solutions are mixed.

Three c.c. of concentrated aqueous solution of permanganate of potash are put into a porcelain capsule (one part of permanganate dissolves in 16 of water at 15° C.). The hæmatoxylin solution is added, and the whole is gradually heated up to boiling-point with constant stirring. The solution turns to a dark reddish violet. The boiling is continued for half a minute or a minute. The solution is then cooled, which is best done by floating the capsule on to cold water. It may be filtered after cooling if desired, but this is seldom necessary. The solution is now ripe and ready for use.

The object of the permaganate in this process is of course to effect the instantaneous oxidation of the hæmatoxylin. Hansen fancies that it has another point of utility: that by destroying all germs that may be present in the ingredients it should make the solution keep better than the usual solutions. As a matter of fact it does not; mine formed a pellicle and strong precipitate in a few days, and the same was found at the Naples station (MAYER, *in litt.*).

With this exception I find the solution excellent; the stain is most beautiful, and cannot be distinguished from that of hæmalum.

MAYER (*Mitth. Zool. Stat. Neapel*, xii, 2, 1896, p. 309) thinks the presence of the manganese in the solution favours the formation of the precipitate. He suggests that the oxidation might be brought about by taking an *alcoholic* solution of hæmatoxylin, leaving it for a short time in contact with the permanganate, and then adding it to 5 per cent. alum solution. The alcohol would then not bring over manganese into the stain.

253. Böhmer's Hæmatoxylin (*Arch. f. mik. Anat.*, iv, 1868, p. 345; *Aerzt. Intelligenzbl., Baiern*, 1865, p. 382).—Make (A) a solution of hæmatox. cryst. 1 part, alcohol (absolute) 12 parts, and (B) alum 1 part, water 240. For staining, add two or three drops of A to a watch-glassful of B.

The staining solution ought not to be made up at the moment of using, but should be made up beforehand and allowed to ripen for some days (Mayer says at least two months). The alcoholic solution of hæmatoxylin may be kept in stock; it becomes brown, but does not lose its properties.

Washing out may be done with a 0·5 per cent. solution of alum in water, or with acids if desired.

I consider this to be of merely historical interest.

254. Delafield's Hæmatoxylin (*Zeit. f. wiss. Mik.*, ii, 1885, p. 288).—The history of this formula is as follows:—It had long been in use in the Institute of Pathology at Heidelberg, when it was communicated by Pfitzner to Flemming, who published it and particularly recommended it in his *Zellstz.*, &c., p. 388, 1882. Flemming then attributed the formula to Grenacher, and in consequence the stain went for years by the name of "Grenacher's hæmatoxylin." Later on Flemming discovered that this attribution was erroneous, and attributed the formula to Prudden; and in consequence it was thenceforth known for some time as "Prudden's hæmatoxylin." In 1885 matters were set right by Prudden's explaining that the stain was the invention of Delafield, and publishing the correct formula here quoted (in the formula as published by Flemming the proportions are somewhat different).

To 400 c.c. of saturated solution of ammonia-alum* add 4 grms. of hæmatox. cryst. dissolved in 25 c.c. of strong alcohol. Leave it exposed to the light and air in an unstoppered bottle for three or four days. Filter, and add 100 c.c. of glycerin and 100 c.c. of methylic alcohol (CH_4O). Allow the solution to stand until the colour is sufficiently dark, then filter and keep in a tightly stoppered bottle.

---

* Ammonia-alum dissolves in about 11 parts of water.

This solution keeps well,—it may be said to keep for years. It is well to allow it to ripen for at least two months before using it.

For staining, enough of the solution should be added to pure water to make a very dilute stain; and even then care should be taken not to leave objects too long in the fluid. It is an extremely powerful stain.

BÜTSCHLI (*Unters. üb. mikroscopische Schäume u. das Protoplasma*, &c., 1892; *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 197) recommends, under the name of "acid hæmatoxylin," solution of Delafield very strongly diluted, and with enough acetic acid added to it to give it a decidedly red tint. This gives a sharper and more differentiated nuclear stain than the usual solution.

I used Delafield's solution a good deal a few years ago, in view of its faculty for affording a plasma-stain at the same time as a nuclear stain. It sometimes gives good images of spindles, &c., but the stain is very opaque. There are several better plasma-stains known now, and I should suppress it were it not that I see that it is still used by some good workers, Flemming amongst the number. It may perhaps still be useful for staining tissues that have been overcharged with metallic salts during fixation or hardening, and are therefore unusually difficult to stain.

255. Ehrlich's Acid Hæmatoxylin (*Zeit. f. wiss. Mik.*, 1886, p. 150).—

Water	100 c.c.
Absolute alcohol	100 ,,
Glycerin	100 ,,
Glacial acetic acid	10 ,,
Hæmatoxylin	·2 grms.
Alum in excess.	

Let the mixture ripen in the light (with occasional admission of air) until it acquires a dark red colour. It will then *keep*, with constant staining power, *for years*, if kept in a well-stoppered bottle. Sections are stained in a few minutes. It is stated that the solution is also very appropriate for staining in bulk, as over-staining does not occur; but this I take leave to doubt.

Of all the old formulæ I have tried, this is the one that has given me the sharpest chromatin stain.

MANN (*Zeit. f. wiss. Mik.*, xi, 4, 1895, p. 487) makes up this stain with hæmatein instead of hæmatoxylin.

256. Ammoniated Hæmatoxylin (SQUIRE, *Methods and Formulæ*, &c., p. 24).—"Hæmatoxylin, 2 grms.; ammonium carbonate, 0·4 grm.; proof spirit, 40 c.c. Dissolve the ammonium carbonate and the hæmatoxylin in the proof spirit, and expose to the air in a shallow dish for twenty-four hours; make up the volume to 40 c.c. with proof spirit (warming if necessary to dissolve any separated crystals), and add ammonia alum, 2 grms., dissolved in distilled water, 80 c.c.; glycerin, 100 c.c.; rectified spirit, 80 c.c.; glacial acetic acid, 10 c.c. This solution requires no 'ripening,' and is ready to be diluted for use (1—10)."

I apprehend that the statement that the solution requires no ripening is erroneous. I find the solution precipitates on dilution, and although it gives a fine stain, is therefore disagreeable to work with. It has this good quality, that it keeps extremely well.

257. MAYER'S **Hæmacalcium** (*Mitth. Zool. Stat. Neapel*, x, 1, 1891, p. 182).—Hæmatein (or hæmateate of ammonia, §§ 245, 246), 1 grm.; chloride of aluminium, 1 grm.; chloride of calcium, 50 grms.; glacial acetic acid, 10 c.c. (or common acetic acid, 20 c.c.); 70 per cent. alcohol, 600 c.c. Rub up finely together the first two ingredients, add the acid and alcohol, dissolve either cold or with heat; lastly add the chloride of calcium.

A reddish-violet liquid, imagined as a substitute for KLEINENBERG'S solution, in view of the many disadvantages of the latter (see below). To be used in the main as Kleinenberg's. If the objects stain in too red a tone they should be treated with a solution (of about 2 per cent.) of chloride of aluminium in alcohol, or with a 0·5 to 1 per cent. solution of acetate of soda or potash in absolute alcohol, or with bicarbonate of soda (see above, § 247).

The solution is not perfectly stable, but in course of time (*Mitth. a. d. Zool. Stat. Neapel*, x, 3, 1892, p. 499) turns blue and precipitates. To avoid this the mixture should be made up in two separate bottles, each containing half of the alcohol and of the acid, and one containing besides all the calcium chloride, the other all the hæmatein and all the aluminium chloride, equal quantities being taken from each when required for staining.

With certain objects this solution does not penetrate well, the stain being confined to their superficial parts. This may be remedied by acidifying the solution, or, which is better, by leaving the objects for some time before staining in acid alcohol. Anyway objects ought NOT to have an alkaline reaction. If these precautions be taken, it will not be necessary to use acid for washing out. For some objects also (*e. g.* Hydroida) the penetrating effect is enhanced by diluting the solution with one third volume of glycerin, or by increasing the proportion of aluminium chloride up to about eight times that of the hæmatein.

The solution is not recommended as giving as good results as hæmalum,—as a stain it is distinctly inferior; and Mayer is of opinion that no alcoholic hæmatein solution can be made to give as precise a stain as the aqueous solutions, for he holds that the watery nature of the menstruum is of itself of a beneficial effect so far as regards the *quality* of the stain. He recommends it merely as a substitute for Kleinenberg's (in cases in which an alcoholic hæmatein stain seems indicated), as being convenient, easy to prepare, and constant in its effects, none of which qualities belong to Kleinenberg's formula.

258. Kleinenberg's Hæmatoxylin (*Quart. Journ. Micr. Sci.*, lxxiv, 1879, p. 208).—Highly irrational and very inconstant in its composition and its effects. For a detailed account of the reasons for which I hold that this solution should be discarded, see last edition; also the elaborate criticism of MAYER (*Mitth. a. d. Zool. Stat. zu Neapel*, 10, 1, 1891, p. 174), and that of SQUIRE, in his *Methods and Formulæ*, p. 25.

The alternative formulæ of SQUIRE (*l. c.*) and of VON WISTINGHAUSEN (*Mitth. Zool. Stat. Neapel*, x, 1891, p. 41; *Zeit. f. wiss. Mik.*, x, 4, 1893, p. 479) do not appear to get over the difficulty of realising the production of the required proportion of aluminium chloride by the double decomposition of alum and calcium chloride.

259. Other Alumina-hæmatein Lakes.—RANVIER'S **Hæmatoxylin** (*Comptes rend. Ac. Sc.*, 2 sem., t. xcv, p. 1375). CONTEJEAN (*Bull. Soc. Philomath. Paris*, 8, 3, 1891, p. 117). (Cf. Mayer, in *Mitth. a. d. Zool. Stat. zu Neapel*, 10, 3, 1892, p. 500.) Critically examined, these methods appear to be only roundabout methods of getting a solution of hæmatein in alum. RENAUT'S **Glycerin Hæmatoxylin** (**"Glycérine Hématoxylique,"** *Arch. de. Physiol.*, 1881, p. 640). **Iodine Hæmatoxylin** (CUCCATI, *Zeit. f. wiss. Mik.*, v, 1, 1888, p. 55). **Iodine Hæmatoxylin** (SANFELICE, *Journ. de MicrOgr.* xiii, 1889, p. 335; *Journ. Roy. Mic. Soc.*, 1889, p. 837). Mayer (*Mitth. Zool. Stat. Neapel*, 10, 1, 1891, pp. 178, 182) remarks on this that it is essentially a Böhmer's solution with somewhat more alcohol. GAGE'S chloral solution (*Proc. Amer. Mic. Soc.*, 1893, p. 125; *Journ. Roy. Mic. Soc.*, 1893, p. 564); the addition of chloral is supposed to make the solution keep, but does not (MAYER, *in litt.*). HACKE'S, *Compt. rend. Soc. Biol. Paris*, x, 1, 1894, pp. 253—369 (complicated and superfluous, MAYER). And for some other superfluous formulæ see previous editions.

B. *Other Hæmatein Lakes.*

260. R. HEIDENHAIN'S Hæmatoxylin (*Arch. f. mik. Anat.*, 1884, p. 468, and 1886, p. 383).—Stain for twelve to twenty-four hours in a $\frac{1}{3}$

per cent. solution of hæmatoxylin in pure water (distilled water only should be used). Soak the objects for the same length of time in a 0·5 per cent. solution of neutral chromate of potash, which should be changed, if necessary, several times. Wash out the excess of chromate with water, and treat further as desired.

The above is a slightly modified form of the original process, in which staining was done in a stronger hæmatoxylin solution (0·5 to 1 per cent.), and bichromate was used for washing out instead of neutral chromate. The more recent process gives a sharper chromatin stain.

The stain succeeds best with alcohol or picric acid objects, but it will succeed with chromic objects if they have been very well washed, or with material fixed in Flemming's mixture.

Objects that have been fixed in corrosive sublimate ought to be very carefully washed out with water (many hours in running water), as neutral hæmatoxylin forms a black precipitate with the excess of sublimate that remains after washing out with alcohol (see TORNIER, in *Arch. f. mik. Anat.*, 1886, p. 181). To avoid this precipitate it is well to stain in the dark.

The stain is black to grey (hæmatoxylin forming with chromic salts a black compound). It is a sharp stain, remarkably rich in detail.

The process is *adapted to staining in bulk.* Perhaps its greatest advantage lies in the fact that you can decolour the objects to any extent by prolonging the washing in the chromate.

The method may be varied by washing out after staining with alum solution (1 per cent.) instead of a chromate. In this case the stain will be blue.

This is not, so far as I can see, as fine a stain as M. HEIDENHAIN'S iron-hæmatoxylin, but it has the advantage of being applicable to staining in bulk, which the latter is not. It is *a plasma-stain as well as a chromatin stain.*

261. APÁTHY'S **Modification of Heidenhain's Process** (*Zeit. f. wiss. Mik.*, v, 1, 1888, p. 47).—This is an *alcoholic* method. Stain in a 1 per cent. solution of hæmatoxylin in 70 or 80 per cent. alcohol. Wash out (for "thin" sections, *i. e.* sections of 10 to 15 μ, half the time of staining—for "thicker" sections of 25 to 40 μ twice the time of staining) in 1 per cent. solution of bichromate of potash in 70 to 80 per cent. alcohol.

The bichromate solution is conveniently prepared by mixing one part of a 5 per cent. aqueous solution with about four parts of 80 to 90 per cent. alcohol. The mixture should be made immediately before using, and should be kept from the light (light precipitates it) during the process of decoloration, and should also be changed for fresh several times during the process. After the differentiation of the colour has been accomplished, the objects should be thoroughly washed (still in the dark) in several changes of 70 per cent. alcohol.

Preparations made in this manner are said to be more transparent and better preserved than those made by Heidenhain's process.

For staining celloidin series of sections, Apáthy also (*Zeit. f. wiss. Mik.*, vi, 2, 1889, p. 170) recommends the following procedure:—Stain in the hæmatoxylin solution as above for ten minutes; then remove the excess of hæmatoxylin fluid from the sections by means of blotting-paper, and bring the series for five to ten minutes into 70 per cent. alcohol containing only a few drops of a strong (5 per cent.) solution of bichromate. This must be done in the dark. If the hæmatoxylin be not removed with blotting-paper as described, the celloidin will take the stain. The sections should appear steel-blue to steel-grey.

262. Iron Hæmatoxylin (M. Heidenhain, "Über Kern und Protoplasma," in *Festschr. Herrn Geheimr. A. v. Kölliker, &c., gewidm.*, 1892, p. 118; *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 204).—This process is based on an old method, now given up, of Benda (*Verh. Phys. Ges. Berlin*, 1885–6, Nos. 12, 13, 14; *Arch. f. Anat. u. Phys.*, 1886, p. 562). Benda mordanted as here in ferric alum, but differentiated in chromic acid. Heidenhain's process is as follows:—Sections are treated from half an hour to at most two or three hours with a 1·5 to 4 per cent. solution of ferric alum. By this is meant the double salt of the sesquioxide of iron $(NH_4)_2Fe_2(SO_4)_4$, in clear violet crystals; the double salt of the protoxide, or salt of Mohr in green crystals, will not serve. If the crystals have become yellow and opaque, they have gone bad, and should be rejected. They ought to be kept in a stoppered bottle, the solution should be made in the cold (*Arch. f. mik. Anat.*, xliii, 3, 1894, pp. 431, 435). The sections are then washed with water and stained for half an hour in an aqueous solution (of about 0·5 per cent.) of hæmatoxylin. (Hæmatoxylin is stated by Heidenhain to give better results than hæmatein.) They are then rinsed with water, and again treated with the iron solution, which slowly washes out the stain. The progress of the differentiation ought to be controlled under the microscope. The sections should to this end be removed from time to time from the alum solution, and put into tap water whilst they are being examined. This is favorable to the stain. I find my sections require from twenty minutes to an hour or more for satisfactory differentiation. As soon as a satisfactory differentiation has been obtained, the preparations are washed for at least a quarter of an hour in running water, but not more than an hour, and mounted. The results differ, in Heidenhain's view, according to the duration of the treatment with the iron and the stain. If the baths have been of short duration, viz. not more than half an hour in the iron and as much in the stain, *blue* preparations will be obtained. These show a very intense and highly differentiated stain of all nuclear structures, achromatic as well as chromatic, cytoplasmic structures being pale. If the baths in the iron and in the stain have been prolonged (twelve to eighteen hours), and the subsequent differentiation in the second iron bath also duly prolonged, *black* preparations will result. These show chromosomes stained, central and polar bodies stained intensely black, cytoplasm sometimes colourless, sometimes grey, in which case achromatic spindle-fibres and cell-plates are stained, connective-tissue fibres black, red blood-corpuscles black, micro-organisms sharply stained, striated muscle very finely shown.

Dr. MAYER, writing to me, doubts that the blue or black tone is conditioned by the duration of the mordanting and staining baths; and my observations confirm this view.

I most highly recommend this stain, which is one of the very finest I am acquainted with. It may be used either with sublimate or alcohol material, or after liquid of Flemming. The process is extremely easy to manage. It is *only applicable to sections,* which should be thin, not more than 8 μ in thickness. The preparations are said to be perfectly permanent. It has been said that this process frequently gives rise to amorphous precipitates in the tissues. I find that it does sometimes, but not to any very injurious extent.

A modification of this process, consisting in a foregoing stain with Bordeaux, which is said to enhance its power of demonstrating centrosomes, will be given under "Cytological Methods."

263. BENDA'S **later Sulphate of Iron Hæmatoxylin** (*Verh. d. Anat. Ges.*, vii, 1, 1893, p. 161; *Zeit. f. wiss. Mik.*, xi, 1, 1894, p. 69; RAWITZ, *Leitfaden*, 1895, p. 73).—This is a later form of the old method of BENDA mentioned in the last section. BENDA found that the ferric alum of that process gave rise to amorphous precipitates in the tissues. To avoid them he now proceeds as follows:—Tissues fixed in any way may be employed. Sections are mordanted for twenty-four hours in *Liquor ferri sulfurici oxidati,* P. G., diluted with one or two volumes of water (this preparation consists of sulphate of iron 80 parts, water 40, sulphuric acid 15, and nitric acid 18, and contains 10 per cent. of Fe). They are then well washed, first with distilled water, then with tap water, and are brought into a 1 per cent. solution of hæmatoxylin in water, in which they remain till they have become thoroughly black. They are then washed and differentiated. The differentiation may be done either in 30 per cent. acetic acid, in which case the progress of the decoloration must be watched; or in a weaker acid, which will not require watching; or in the sulphate solution strongly diluted with water.

This process is said to be applicable to all sorts of organs, and to give in particular excellent images of axis-cylinders, and of the achromatic figure of cell-division. For this latter object it may be useful to add a second stain with Säurefuchsin. Chromatin, centrosomes and spindle relics (*Zwischenkörper*) should then appear black; linin fibrils and spindle-fibres red. I find it to be a powerful and sharp stain, but the strong iron solution is a disagreeable reagent to handle. If the iron solution be taken for the differentiation, it should be taken extremely diluted (of a *very pale* straw-colour), and the progress of the differentiation watched; as if it be only diluted about tenfold, for instance, the decoloration is extremely rapid.

264. **Iron Hæmatoxylin** (BÜTSCHLI, *Unters. über mikroskopische Schäume u. das Protoplasma*, &c., 1892; *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 197).—Sections treated with a weak brown aqueous solution of ferric acetate, washed with water, and stained in 0·5 per cent. aqueous solution of hæmatoxylin. This treatment gives a blue-black or brown-black stain of extraordinary intensity. The process was used by Bütschli for staining sections, 1 μ in thickness, of Protozoa. It does not appear to be of general applicability.

265. **Benda's Copper Hæmatoxylin** (*Arch. f. mik. Anat.*, xxx, p. 49).—Fix in Flemming. Make paraffin sections. Stain them as follows:—Put them for twenty-four hours into concentrated solution of neutral acetate of copper, kept at a temperature of about 40° C., or for forty-eight hours at the normal temperature. Wash them out well with water, and stain for a few minutes, or to a dark grey or black tint, in 1 per cent. aqueous hæmatoxylin solution. Decolourise in dilute hydrochloric acid (0·2 per cent.), until the sections appear of a fairly light yellow. You decolourise more or less according as you wish to have a more or less plasmatic stain or a purely nuclear one. The acid must now be neutralised, which may be done by putting the sections back into the copper solution until they turn of a bluish grey. Then wash, dehydrate, and mount in balsam. See also the remarks of PIERSOL, in *Amer. Mon. Mic. Journ.*, 1887, p. 155; or in *Journ. Roy. Mic. Soc.*, 1888, p. 158; or *Zeit. f. wiss. Mik.*, v, 4, 1888, p. 499. This process gives an energetic differentiation of karyokinetic

figures, and is said to be particularly applicable to the study of spermatogenesis. It seems to me to be not very easy to manage, as the proper degree of differentiation ought to be hit off to a nicety, which is rather a difficult matter.

266. Mallory's **Phospho-molybdic Acid Hæmatoxylin** (*Anat. Anzeig.*, 1891, p. 375; see also *Zeit. f. wiss. Mik.*, viii, 3, 1891, p. 341).

267. Minot's **Hæmatoxylin Methods** (*Zeit. f. wiss. Mik.*, iii, 2, 1886, p. 177).

CHAPTER XV.

ON STAINING WITH COAL-TAR COLOURS.

268. Basic, Acid and Neutral Coal-tar Colours.—Histologists generally conceive of the coal-tar colours as divided into three groups, according to a principle of classification founded on chemical considerations, and introduced into histological literature by Ehrlich (*Verb. d. Berl. Phys. Ges.*, May 16th, 1879; in Reichert and Du-Bois Reymond's *Arch. f. Anat. u. Phys., phys. Abth.*, 1879, p. 571). These three groups are those of the *basic* colours, the *acid* colours, and the *neutral* colours. By a "basic" colour is meant a compound in which the colouring principle or molecular group to which the compound owes its colouring properties exists as or chemically plays the part of a base combined with a colourless acid. For instance, fuchsin or magenta is a basic colour. It is the hydrochloride of rosanilin, and its colouring properties are due to the rosanilin which exists as a base in the compound, and not to the hydrochloric acid of the compound. By an "acid" colour is meant a compound in which the colouring principle exists as or plays the part of an acid. The dye known as acid fuchsin or acid magenta (Säurefuchsin) is an acid colour. It is the soda salt of di- or tri-sulphoconjugated rosanilin, that is of rosanilin di- or tri-sulphonic acid, and its colouring properties are due to the rosanilin which exists as an acid in the compound, and not to the soda. Or to take a simpler case, picrate of ammonia is an acid colour in Ehrlich's sense, and its colouring properties are evidently due to the picric acid in it, and not to the ammonia. The neutral colouring matters form a very small group; the only example that I can find mentioned in Benedikt and Knecht's *Chemistry of the Coal-tar Colours* being artificial indigo, obtained from propiolic acid. Ehrlich holds that neutral colours are, however, frequently formed by the mixture of the solutions of an acid colour and a basic colour. They are generally insoluble in pure water, and hence precipitate when the mixture is made, but may be got to re-dissolve by adding an excess of the acid colour.

Now, according to Ehrlich, the basic colours are in general chromatin stains,—that is, they have a special affinity for the element of nuclei known as chromatin, so that they are mostly sharp nuclear stains. The acid colours, on the other hand, are, according to him, in general plasma stains,—that is, they have a special affinity for cytoplasm and intercellular substances. And lastly, the neutral colours exhibit special affinities for certain cell-contents; amongst them are found some important granule stains.

I would not for a moment impugn the accuracy or thoroughness of the observations on which this generalisation is based, or its utility from a theoretical point of view; but I must say that as a general histological classification of tar colours it requires to be supplemented by a good deal of explanation and restriction. In practical histology we have to take account not only of the affinities of a dye for this or that cellular element, as they are manifested in *progressive* staining under narrowly limited conditions: we have also to take account of the resistance of the stain to the liquids employed for washing, for dehydration, for clearing; in short, we have to take into account the way in which the dye behaves when employed as a *regressive* stain. This is of peculiar importance in the case of the coal-tar colours, for their principal use is for the *regressive* staining of sections destined to be dehydrated by alcohol and mounted in balsam. Now Ehrlich's experiments take no account of these conditions. He worked with "cover-glass preparations" of isolated cells, such as blood and lymph cells, and was thus able to avoid the prolonged washing necessary for most sections, and to suppress altogether the dehydration by alcohol, his cover-glass preparations being simply dried, after staining, in a stove. In consequence, his chemical categories of *basic colours* and *acid colours* fail to correspond precisely to the technical categories of *chromatin stains* and *plasma stains.*

For instance, orange is an acid colour; but used as a regressive stain I find it will give a very sharp stain of chromatin and plasmatic nucleoli: it cannot, therefore, be classed as a mere plasma stain, though it is also a very good plasma stain. Säurefuchsin is also an acid colour. It behaves in general as a decided plasma stain. But used as a regressive stain it sometimes, under conditions which I am not able to specify, gives a very vigorous stain of chromatin. Safranin is a basic colour, but by the use of appropriate mordants it can be made to behave as a plasma stain. Methylen blue is a basic colour. But, as is well known, when employed according to the method worked out by Ehrlich for the so-called *intra-vitam* staining of nerves, it affords a stain that is essentially plasmatic, such staining of nuclei as may occur in this process being an accidental epiphenomenon. Nigrosin is, according to Ehrlich, an acid colour, and should therefore be essentially a plasma stain. Yet I find that, used as a regressive stain in the same way as safranin, it gives a vigorous chromatin stain, cytoplasm being only faintly coloured. Bordeaux is an acid colour, but it stains chromatin as well as cytoplasm.

It would seem, therefore, that Ehrlich's generalisation, however important it may be from a theoretical point of view, does not hold good as a statement of the behaviour of tar colours when employed for staining sections in the usual way. It is roughly true that the basic colours are in general chromatin stains, and the acid colours in general plasma stains; but the rule is subject to many exceptions.

269. Progressive and Regressive Coal-tar Stains.—Very few anilins give a precise nuclear or chromatin stain by the *progressive* or *direct* method (§ 211). Two of them—methyl green and Bismarck brown—are pre-eminently chromatin stains. Many of the others—for instance, safranin, gentian, and especially dahlia—may be made to give a nuclear stain with fresh tissues by combining them with acetic acid; but in ninety-nine cases out of a hundred are not so suitable for this kind of work as the two colours first named, which practically form a class apart.

Again, very few anilins give a pure plasmatic stain (one leaving nuclei unaffected). The majority give a diffuse stain, which in some few cases becomes, by the application of the *regressive* or *indirect* method (§ 211), the most precise and splendid chromatin stain as yet obtainable by any means.

The regressive staining method, or Flemming's method, will form the subject of the present chapter, and the anilin chromatin stains will be treated of in the next chapter, the anilin plasma stains being reserved for treatment in a later chapter.

*General Directions for the Regressive Staining Method, as applied to Coal-tar Colours.**

270. Staining.—*Sections only,* or material that is thin enough to behave like sections, such as some membranes, can be stained by this method.

The solutions employed are made with alcohol, water, or anilin, according to the solubility of the colour. There seems to be no special object in making them with alcohol if water will suffice, the great object being to get *as strong a solution as possible.* Alcohol of 50 per cent. strength, however, may be said to constitute a very generally desirable medium. The sections must be *very thoroughly* stained in the solution. As a general rule they cannot be left too long in the staining fluid. With the powerful solutions obtained with anilin a few minutes or half an hour will frequently suffice, but to be on the safe side it is frequently well to leave the sections twelve to twenty-four hours in the fluid. Up to a certain point the more the tissues are stained the better do they resist the washing-out process, which is an advantage. For researches on nuclei the solutions made with anilin had better be employed *only* with preparations well fixed in chromo-aceto-osmic acid, as the basic anilin oil may easily attack chromatin if not specially well fixed.

Material fixed in chromo-osmic mixtures gives a sharper and more selective stain than material fixed in sublimate or the like. During the staining the tissues become *overstained,* that is charged with colour in an excessive and diffuse manner. The stain must therefore now be *differentiated* by removal of the excess of colour.

---

* Historically the principle of this method is due to HERMANN and BOETTCHER; but it is universally known by the name of FLEMMING, to whom is due the credit of having greatly improved the method in its practical details.

271. Differentiation.—This is generally done with alcohol, sometimes pure, sometimes acidulated (with HCl). The stained sections, if loose (celloidin sections), are brought into a watch-glassful of alcohol; if mounted in series on a slide they are brought into a tube of alcohol (differentiation *can* be done by simply pouring alcohol on to the slide, but it is better to use a tube or other bath). It is in either case well to *just rinse* the sections in water, or even to wash them well in it, before bringing them into alcohol.

The sections in the watch-glass are seen to give up their colour to the alcohol in clouds, which are at first very rapidly formed, afterwards more slowly. The sections on the slide are seen, if the slide be gently lifted above the surface of the alcohol, to be giving off their colour in the shape of rivers running down the glass. In a short time the formation of the clouds or of the rivers is seen to be *on the point of ceasing;* the sections have become *pale* and somewhat *transparent,* and (in the case of chrom-osmium objects) have *changed colour,* owing to the coming into view of the general ground colour of the tissues, from which the stain has now been removed. (Thus chrom-osmium-safranin sections turn from an opaque red to a delicate purple.) At this point the differentiation is complete, and the extraction of the colour *must be stopped instantly* (see § 273).

It is generally directed that absolute alcohol be taken for differentiation. This may be well in some cases, but in general strong (95 per cent.) spirit is found to answer perfectly well.

The hydrochloric acid alcohol process had better only be employed with tissues well fixed with "Flemming," as with tissues imperfectly fixed it may cause swellings. Further, the acid extracts the colour much more quickly from resting nuclei than from kinetic nuclei, which is an advantage or a disadvantage according to the end in view.

The proportion of HCl with which the alcohol should be acidified for the acid process should be about 1 : 1000, or less; seldom more.

As a rough and ready guide to the beginner, it may be stated that washing out should be done with pure alcohol whenever it is desired to have resting nuclei stained as well as dividing nuclei; the other processes serving chiefly to differentiate mitoses.

Differentiation with pure alcohol is known as "neutral differentiation," or "neutral extraction;" and differentiation with hydrochloric acid is known as "acid differentiation," or "acid extraction."

The length of time necessary for differentiating to the precise degree required varies considerably with the nature of the tissues and the details of the process employed; all that can be said is that it generally lies between thirty seconds and two minutes. The acid process is vastly more rapid than the neutral process, and therefore of course more risky.

In more than one of the methods presently to be described treatment with chromic acid or with iodine forms part of the differentiating process. The *rationale* of this is somewhat obscure; the most probable point of view appears to be that the chromic acid acts as a mordant on the chromatin, and helps it to retain the stain. It is known on the one hand that chromic acid precipitates safranin from its solutions, so that by admitting a special affinity of chromic acid on the other hand for chromatin, and especially for chromatin in the kinetic state, the explanation is hypothetically complete.

The iodine in Bizzozero's (Gram's) process also appears to act as a fixative of the colour. See in the next chapter the special processes of differentiation described under *Safranin* and *Gentian Violet.*

272. Substitution.—It was stated above that differentiation is generally done with alcohol. There exists another mode of differentiation that is both of practical importance and of theoretical interest—one anilin stain may be made to wash out another. Thus methylen blue and gentian violet are discharged from tissues by aqueous solution of vesuvin or of eosin: fuchsin is discharged from tissues by aqueous solution of methylen blue. The second stain "substitutes" itself for the first in the general "ground" of the tissues, leaving, if the operation have been successfully carried out, the nuclei stained with the first stain, the second forming a "contrast" stain.

Flemming obtains a highly important stain by differentiating in a solution of Orange G sections that have been previously stained with gentian violet (see his Orange method in the chapter on Plasma Stains). Flemming attributes the differentiation in this case to the "acid" qualities of the Orange. But it should be borne in mind that Orange G is an acid colour in two senses of the word; not only is it an acid colouring matter in Ehrlich's sense, but its solution in water has an acid reaction; and it would seem possible that it is rather to this than to its "acid" composition in the sense of molecular structure that its power of extracting gentian violet is due. I am not able to say how far the "acid" nature of dyes in Ehrlich's sense confers on them the power of extracting the stains of basic colours, or of less acid colours. It is certain at any rate that this property is also possessed by some basic colours, as is testified by two of the examples given above, both vesuvin and methylen blue being basic colours.

In the interesting paper of RESEGOTTI in *Zeit. f. wiss. Mik.*, v, 3, 1888, p. 320, it is stated as a very general rule that colours that do *not* give a nuclear stain by the regressive method will wash out those that *do*. Thus he found that—

Safranin, Dahlia, Methyl Violet, Gentian Violet, Rubin, Victoria Blue, Magenta, Basic Fuchsin, are washed out by the following:

Congo, Methyl Green, Iodine Green, Nigrosin, Methylen Blue, Orange, Ponceau, Acid Fuchsin, Aurantia, Cyanin, Eosin, Methylic Eosin, Magdala Red, Bordeaux, Vesuvin. But RESEGOTTI's experiments do not seem to me to constitute a case in point. For he used the second colour, if I understand him rightly, in *alcoholic* solution; so that it remains uncertain how far the differentiation should be attributed to the chemical nature of the second colour, and how far to the alcohol used as a vehicle. The same remark applies to BENDA's Safranin-and-Lichtgrün process.

273. Clearing.—The differentiation having been carried to a satisfactory point, as described in § 271, the extraction of the colour may be stopped by putting the sections into water; but the general practice is to clear and mount them at once.

You may clear with clove oil, *which will extract some more colour* from the tissues. Or you may clear with an agent that does not attack the stain (cedar oil, bergamot oil, xylol, toluol, naphtha, &c.; see the chapter on Clearing Agents). If you have used pure alcohol for washing out, you had perhaps better clear with clove oil, as pure alcohol does not always, if the staining have been very prolonged, extract the colour perfectly from extra-nuclear parts. But if you have not stained very long, and if you have used acidulated alcohol for washing out, clove oil is not necessary, and it may be better not to use it, as it somewhat impairs the brilliancy of the stain. A special property of clove oil is that it helps to differentiate karyokinetic figures, as it *decolours resting nuclei more rapidly than those in division.*

Some colours are much more sensible to the action of clove oil than others; and much depends on the quality of this much-adulterated essence. New clove oil extracts the colour more quickly than old.

Series of sections on slides are conveniently cleared by pouring the clearing agent over them.

When the clearing is accomplished to your satisfaction, mount in damar or balsam, or stop the extraction of the colour if clove oil have been used by putting the sections into some medium that does not affect the stain (xylol, cedar oil, &c.). Chloroform should be avoided, either as a clearer or as the menstruum for the mounting medium.

274. General Results.—The results depend in great measure on the previous treatment of the tissues. If you have given them a prolonged fixation in Flemming's *strong* chromo-aceto-osmic mixture, and have differentiated after staining with acid alcohol and cleared with clove oil, you will get, with some special exceptions, nothing stained but nucleoli and the chromatin of dividing nuclei, that of resting nuclei remaining unstained. If you have given a lighter fixation, with Flemming's weak mixture or some other fixing agent not specially inimical to staining, and have differentiated after staining with pure alcohol, you will get the chromatin of resting nuclei stained as well.

275. HENNEGUY's **Permanganate Method** (*Journ. de l'Anat. et de la Physiol.*, xxvii, 1891, p. 397).—This method is based on the fact, discovered by HENNEGUY, that permanganate of potassium is a mordant for many anilin dyes, and will enable a good stain to be procured in cases in which the usual methods fail.

Sections (from tissues fixed either in the strong solution of Flemming for from two to six hours, or in other reagents such as sublimate, liquid of Perenyi, liquid of Kleinenberg, alcohol, &c.) are treated for five minutes with 1 per cent. solution of permanganate of potassium. They are then washed with water and stained (for about half the time that would have been taken if they had not been mordanted with the permanganate) in safranin, rubin, gentian violet, vesuvin, or the like. The stain that succeeds the best is a safranin solution prepared with anilin water and absolute alcohol (see below, § 278). After staining they are differentiated with alcohol, followed by clove oil in the usual way. This is the delicate part of the process. The progress of the decoloration must be watched under the microscope, in order that it may be stopped at the proper moment. It goes on in general slowly, and the slower it proceeds the more selective will be the resultant stain. The decoloration sometimes continues even after the sections have been mounted in balsam, especially if all traces of clove oil have not been removed before mounting. It may thus happen that preparations which are insufficiently washed out at the moment of mounting show a perfectly differentiated stain twenty-four or forty-eight hours afterwards.

If safranin have been taken as the stain the preparations show protoplasm of an orange-grey tint, which shows up the most delicate structures, in particular the achromatic figures of Cytodieresis. Chromosomes and nuclear membranes are of a brilliant red, attractive spheres and centrosomes less strongly stained, but still sharply brought out against the rest of the cytoplasm.

If it be desired to have the details of protoplasmic structures still more markedly brought out, the safranin stain may be *preceded* by hæmatoxylin staining (0·5 per cent. solution of hæmatoxylin in 90 per cent. alcohol ten minutes, wash with water, treat for ten minutes with 2 per cent. solution of bichromate of potash, and wash with distilled water before treating with the permanganate). But with preparations that have been fixed in Flemming this treatment will hardly be necessary.

The mordanting action of permanganate of potassium on anilin stains is so energetic that if it have been overmuch prolonged before staining with safranin, or, still more, with rubin, it becomes almost impossible to wash out the sections properly; it may be necessary to leave them for nearly a month in clove oil.

Henneguy's preparations are certainly most successful, but I do not myself think that the chief value of the process lies in the plasma stain obtained by means of it, though this may be useful for some purposes. I think rather that it may be found useful as a means of slowing the sometimes inconveniently rapid decoloration of sections treated by the regressive method.

276. Ohlmacher's Formaldehyde Process (*Medical News*, February 16th, 1895).—Ohlmacher has found that formaldehyde is a powerful mordant for tar colours. Tissues may either be mordanted separately by treatment for a short time (one minute is enough for cover-glass preparations) with a 2 per cent. to 4 per cent. formalin solution; or the formalin may be combined with the stain. One gramme of fuchsin dissolved in 10 c.c. of absolute alcohol may be added to 100 c.c. of 4 per cent. formalin solution. Or saturated alcoholic solution of gentian violet may be added to 4 per cent. formalin solution in the proportion of 1 : 10. And a like solution may be made by adding to the formalin the like proportion of saturated alcoholic solution of methyl violet 5 B. Or formalin-methylen blue may be made by dissolving 1 grm. of methylen blue in 100 c.c. of the formalin solution. Sections are said to stain in half a minute, and to resist alcohol much more than is the case with those treated by the usual solutions. The formalin solution of safranin (*Safranin O*, from Grübler) is said to give a plasma stain comporting itself in all particulars like eosin.

For the process of Rawitz for adjective staining with tar colours, by which they are made to behave as plasma stains, see the chapter on Plasma Stains.

277. Choice of a Stain.—The tar-colour chromatin stains are sufficiently numerous, so that it almost seems as though we were in presence of an *embarras de richesses*. One might think that it would be quite sufficient for all practical purposes to possess one good red stain and one good blue one, so that, for instance, safranin and thionin or gentian violet

REESE LIBRARY OF THE

should be sufficient for the most exacting of laboratories. But I think it must be admitted that for delicate work, at any rate, it is desirable to possess one or two more. We have to take account of the manner in which these colours behave when used in combination with the plasma stains that it may be desired to employ. And there is another point that is not undeserving of attention. Some of the dyes discussed in the following chapter give a stain of a somewhat dead or dull quality, so much so that chromosomes and nucleoli frequently come out quite opaque. Gentian violet is in this case; whilst dahlia, which is otherwise near to it in hue, is not. Safranin and anilin green, on the other hand, leave the structures beautifully transparent. This is an advantage with thick sections, and sometimes for other reasons; but this transparency of the elements is unfortunately favorable to the production of diffraction lines, which may be a hindrance to good definition in delicate work. So that the dead colours, such as gentian, have a certain advantage for work with very thin sections and where very fine definition of chromatin is required; whilst the transparent or semi-transparent colours, such as safranin, should be preferred for thick sections. I would also add that it always seems to me that the blue stains, such as gentian, are less favorable for work with artificial light. They give more or less dichroic images, which are not favorable to good definition.

The blues seem to have an advantage where it is desired to use them in combination with a plasma stain. For there are many fair plasma stains, such as Säurefuchsin, eosin, orange, in the red or yellow series, but not in the blue series. It is difficult, for instance, to find any plasma-stain except Kernschwarz that will work in a thoroughly satisfactory way with safranin.

To sum up, I would recommend safranin for a red chromatin stain, and thionin or gentian for a blue one, except where special conditions, such as are mentioned above, suggest another choice.

CHAPTER XVI.

THE COAL-TAR CHROMATIN STAINS.

A. *Regressive Stains.*

278. Safranin.—One of the most important of these stains, on account of its great power, brilliancy, and superior permanence in balsam, and also on account of the divers degrees of electivity that it displays for the nuclei and other constituent elements of different tissues.

The great secret of staining with safranin is *to get a good safranin.* It is needful here to insist most urgently on what was said above (§ 216, *sub. finem*). Before thinking of working with this important reagent you should go to Grübler or to Münder and order the safranin you want, specifying whether you want it for staining nuclei or for staining elastic fibres, or for what other purpose you may require it.

There are presumably at least a score of sorts of safranin in the market, differing to a considerable extent in colour, weight, solubility, and histological action. Some are easily soluble in water and not so in alcohol, some the reverse, and some freely soluble in both. Fourteen brands, supplied by Grübler and by Münder, have been studied by RESEGOTTI (*Zeit. f. wiss. Mik.*, v, 3, 1888, p. 320). They all gave positive results with the chromic acid method, to be detailed below; although Grübler had explained that the brands XX, XXBN, TB, had not given positive results (with the usual methods). Resegotti obtained his best results with the brands "Safranin wasserlöslich," "Safranin spirituslöslich," "XX," "XXBN," "TB," furnished by Grübler, and with the brands "Rein," "O," "FII," and "Conc.," supplied by Münder.

The brand I have been using for a long time, and which gives good results, is the "Safranin O" of Grübler and Co. It should be remembered that as the processes of manufacture are constantly changing, the properties of the products are sure to vary somewhat from time to time.

Staining.—The majority of safranins are not sufficiently soluble in water, so that solutions in other menstrua must be employed.

A solution much used some time ago is that of PFITZNER (*Morph. Jahrb.*, vi, p. 478, and vii, p. 291), composed of safranin 1 part, absolute alcohol 100 parts, and water 200 parts, the last to be added only after a few days.

The solution of FLEMMING (*Arch. f. mik. Anat.*, xix, 1881, p. 317) is a concentrated solution in absolute alcohol, diluted with about one half of water.

The solutions of BABES (*Arch. f. mik. Anat.*, 1883, p. 356) are (A) a mixture of equal parts of concentrated alcoholic solution and concentrated aqueous solution (this is very much to be recommended), and (B) a concentrated or supersaturated aqueous solution made with the aid of heat.

Some people still employ simple aqueous solutions.

Lastly, there is the anilin solution of BABES (*Zeit. f. wiss. Mik.*, iv, 4, 1887, p. 470). It consists of water 100 parts, anilin oil 2 parts, and an excess of safranin. The mixture should be warmed to from 60° to 80° C., and filtered through a wet filter. This solution will keep for a month or two.

ZWAARDEMAKER (*Zeit. f. wiss. Mik.*, iv, 2, 1887, p. 212) makes a mixture of about equal parts of alcoholic safranin solution and anilin water (saturated solution of anilin oil in water;—to make it, shake up "anilin oil," which is nothing but pure anilin, with water, and filter). This, I find, will keep for many months, perhaps indefinitely.

Any of these stains may be used with any of the following differentiation processes. Of course you will have to stain longer in the weaker solutions. As to the anilin solutions see *ante*, § 270.

Differentiation.—For general directions for differentiation and clearing see above, §§ 271 and 273.

FLEMMING'S *first method* (or *neutral* differentiation) (l. c. in last section).—Differentiate with pure alcohol, followed by clove oil. This method stains resting chromatin as well as kinetic chromatin.

FLEMMING'S *second method* (or *acid* differentiation) (*Zeit. f. wiss. Mik.*, i, 3, 1884, p. 350).—Differentiate, until hardly any more colour comes away, in alcohol acidulated with about 0·5 per cent. of hydrochloric acid, followed by pure alcohol and clove oil. (You may use the HCl in watery solution if you prefer it.) The strength here given appears unnecessarily high. In Flemming's latest work (on the achromatic structures of the cell) he has been using a lower strength, viz. 0·1 per cent. at most (see *Arch. f. mik. Anat.*, xxxvii, 1891, p. 249); and this I find is generally preferable.

Objects are supposed to have been well fixed—twelve hours at least—in the *strong* chromo-aceto-osmic mixture, and stained for some hours. PODWYSSOZKI (*Beitr. z. path. Anat. v. Ziegler u. Neuwerk*, i, 1886; *Zeit. f. wiss. Mik.*, iii, 3, 1886, p. 405) prefers to stain for half an hour only, and wash out with 0·1 per cent. of HCl in alcohol. In each of these ways you get kinetic chromatin and nucleoli alone stained (if the fixation have been performed as above directed).

PODWYSSOZKI (l. c.) gives another method, which consists in differentiating (for from a few seconds to two minutes) in a strongly alcoholic solution of picric acid, followed by pure alcohol. Same results (except that the stain will be brownish instead of pure red).

BABES employed for differentiating, after staining in the aqueous or alcoholic solutions above mentioned, pure alcohol followed by oil of turpentine. For sections stained in the anilin solution he recommends treatment with iodine, according to the method of GRAM (see what is said as to the process of Gram in the paragraph on gentian violet, next section). This process has also been recommended by PRENANT (*Int. Monatsschr. f. Anat.*, &c., iv, 1887, p. 368), who notes that the treatment with the iodine solution should be somewhat longer, and the treatment with alcohol somewhat shorter than with gentian violet sections.

MARTINOTTI and RESEGOTTI (*Zeit. f. wiss. Mik.*, iv, 3, 1887, p. 328) recommend differentiating with a freshly prepared mixture of one part of 0·1 per cent. aqueous solution of chromic acid with nine parts of absolute alcohol followed by pure alcohol and bergamot oil. In my experience this method does not give better results (I think less good) than that of differentiating by the simple aqueous solution of chromic acid of Bizzozero followed by alcohol (see next section). The latter is certainly a most useful method. It should be mentioned that Martinotti and Resegotti's results refer to lightly stained alcohol-fixed objects, and not to chromo-aceto-osmic objects, which may make a great difference.

GARBINI (*Zeit. f. wiss. Mik.*, v, 2, 1888, p. 170) has recommended that sections be dehydrated, after staining, in methylic alcohol (wood spirit), in which safranin is only very slightly soluble, and decoloured in a mixture of two parts of clove oil with one part of cedar oil. I have not been able to obtain good results by this method.

It has been shown by Ohlmacher (*Journ. Amer. Med. Ass.*, vol. xx, No. 5, Feb. 4, 1893, p. 111) that if tissues be treated with solutions containing iodine or picric acid after staining with safranin, there may be produced in the tissue elements a precipitate of a dark red substance of a crystalline nature, but of lanceolate, semilunar, falciform, or navicellar forms. This precipitate is formed both in normal and pathological tissue, and occurs either in the nuclei or in the cytoplasm. It is formed readily in carcinomatous tissues; and Ohlmacher makes out a strong case in favour of the conclusion to which he has come that many of the bodies that have been described as "coccidia," "sporozoa," or other "parasites" of carcinoma are nothing but particles of this precipitate. This refers, amongst much other work, to that of Podwyssozki and Sawtschenko. Forewarned is forearmed; but if the formation of Ohlmacher's precipitate should prove to be a very general phenomenon, it will be necessary to conclude with him that to follow safranin staining with treatment by solutions containing iodine or picric acid is not only unscientific but positively dangerous. Of course this is not intended to discredit the use of safranin when washed out with hydrochloric acid, alcohol, or the like.

The reader will remember that safranin may be washed out by substitution (see *ante*, § 272).

In preparations made with chromo-aceto-osmic acid, safranin stains, besides nuclei, elastic fibres, the cell-bodies of certain horny epithelia, and the contents of certain gland-cells (mucin, under certain imperfectly ascertained conditions).

279. Gentian Violet.—One of the most important of these stains. It may be used in aqueous solution, or in alcoholic solution diluted with about one half of water (Flemming, *Zells. Kern. u. Zellth.*, 1882, p. 384), and the stain may be differentiated with pure alcohol, or (Flemming, *Zeit. f. wiss. Mik.*, 1, 1884, p. 350) with acidulated alcohol, as directed for safranin. Another good way of using it is that due to Bizzozero (*Zeit. f. wiss. Mik.*, iii, 1, 1886, p. 24). The tissues may be hardened either in alcohol or in a chromic mixture, but must in the latter case have been well washed out with water. The staining solution is borrowed from that of Ehrlich for bacteria, and consists of—

Gentian violet	1 part.
Alcohol	15 parts.
Anilin oil	3 ,,
Water	80 ,,

The sections are stained in it for five or ten minutes or longer (for objects from Flemming's solution it will frequently be advisable to stain for as many hours). After staining, rinse the sections with alcohol, and bring them into a 0·1 per cent. aqueous solution of chromic acid. After from thirty to forty seconds bring them into alcohol, which begins the washing out of the colour. After thirty or forty seconds in the alcohol put them back for thirty seconds into the chromic acid (this is done in order to fix the colour more completely in the nuclei). Then bring them back into alcohol for thirty to forty seconds, in order to wash out more colour and dehydrate them at the same time. Then treat with clove oil, which will extract more colour, and after a short time must be changed for fresh, in which the sections remain until they are seen to give up no more colour, when they are removed and mounted in damar.

You might give a longer treatment with alcohol, and a shorter treatment with clove oil, but you would get a slightly different result. Alcohol washes out colour freely from kinetic nuclei as well as from resting nuclei, whereas clove oil acts much more energetically on the latter than on the former, and thus serves to differentiate dividing nuclei.

In some cases, especially those of tissues whose nuclei have a tendency to give up the colour too freely, better results are obtained by combining the foregoing method with that of Gram for the staining of bacteria (*Fortschr. d. Medicin*, ii, 1884, No. 6; *British Med. Journ.*, Sept. 6th, 1884, p. 486; *Journ. Roy. Mic. Soc.* [N.S.], iv, 1884, p. 817).

In Gram's method the sections are treated, after staining, with a solution composed of—

Iodine	1 gramme.
Iodide of potassium . . .	2 grammes.
Water	300 ,,

In Bizzozero's adaptation of this process the series of operations is as follows:—Stain in the gentian, wash for five seconds in alcohol; two minutes in the iodine solution; twenty seconds in alcohol; thirty seconds in the chromic acid solution; fifteen seconds in alcohol; thirty seconds in the chromic acid again; thirty seconds in alcohol; and treatment with changes of clove oil until final decoloration.

Nissen (*Arch. f. mik. Anat.*, 1886, p. 338) employs this process with omission of the treatment with chromic acid.

In resting nuclei the nucleoli alone are stained, or the chromatin if stained is pale; in dividing nuclei the chromatin is stained with great intensity, being nearly black in the equatorial stage.

This exceedingly powerful stain is quite as precise as that of safranin, to which it is perhaps even preferable for much work with very thin sections (thick sections with closely packed nuclei may easily come out too dark). It lends itself well to double-staining with red or yellow plasma stains.

The stain keeps fairly well in damar, though not so well as that of safranin. Flemming found that after a year it had faded a little, though not so much as hæmatoxylin stains (*v. Zells. Kern. u. Zellth.*, p. 384). My preparations in turpentine-colophonium have kept perfectly for many years, *if not unduly exposed to light.*

Gentian violet in acid solution stains the nuclei of fresh tissues, and dissolved in indifferent media is sometimes very useful for staining *intra vitam* (see above, § 213).

280. Thionin.—The hydrochloride of thionin, or violet of Lauth, is a colour chemically nearly allied to methylen blue. It is, I believe, no longer manufactured for industrial purposes, but may be obtained from Grübler and Co. It was first introduced into histological technique by HOYER, who found it the best of all the special stains of mucin. Later on it was warmly recommended as a chromatin stain by M. HEIDENHAIN. I can thoroughly endorse Heidenhain's recommendation: thionin is about the finest chromatin stain I have seen. I have classed it here as a regressive stain, but its action is so selective from the first that it may almost be considered to be a progressive stain. If you stain for only a short time (a few minutes) in a concentrated aqueous solution, hardly anything but the chromatin will be found to be stained. If the staining be prolonged, plasmatic elements will begin to take up the colour. After a short stain no special differentiation is required; all that is necessary is to rinse with water, dehydrate, and mount. After a strong stain you differentiate with alcohol in the usual way, with this advantage, that the stain is so highly resistent to alcohol that there is no risk whatever of overshooting the mark; the stain will not be more extracted in an hour than that of gentian or dahlia is in a minute, so that the process may be controlled under the microscope if desired. For this reason I think this stain should be specially recommended to beginners. It is a very powerful stain, works well either after sublimate or mixture of Flemming, gives good definition, and seems to keep well.

Henneguy (*in litt.*) says that very good results are got by staining strongly and dehydrating with acetone; but as the differentiation with alcohol is so easy I suppose that this process is only desirable for certain special purposes.

281. Other Regressive Stains.—The foregoing, I think, may suffice for most practical purposes, but the following may be mentioned.

Dahlia (Flemming, *Arch. f. mik. Anat.*, xix, 1881, p. 317).—The stain is paler in the nuclei than with gentian or safranin. The cytoplasmic granulations of certain cells are sharply stained.

Dahlia is also a useful nuclear stain for fresh tissues (*v.* Ehrlich, *Arch. f. mik. Anat.*, xiii, 1876, p. 263). For these the aqueous solution must be acidulated with (five per cent.) acetic acid; or you may stain in a neutral solution, and wash out with acidulated water. Dehydrate with alcohol and mount in turpentine-colophonium. It is also useful for staining *intra vitam* (see above, § 213).

For the staining of Ehrlich's "plasma cells" see *post*, Part II.

Victoria Blue (Victoriablau) (Lustgarten, *Med. Jahrb. k. Ges. d. Aerzte zu Wien*, 1886, pp. 285—291).—I find this to be a very good stain, especially if the sections be previously treated for a few minutes with tincture of iodine.

Victoria has a special affinity for *elastic fibres*. For this object Lustgarten recommends an alcoholic solution of the dye diluted with two to four parts of water. Fixation in chrom-osmium, or at least in a chromic mixture, is, I believe, a necessary condition to this reaction. And you must stain for a long time.

Victoria has also a special affinity for *mucus-cells*, from which it is not washed out by alcohol.

Flemming (*Arch. f. mik. Anat.*, xix, 1881, pp. 317 and 742) mentions also the following:

Magdala Red (Naphthalin Red, Rose de Naphthaline).—Nearly if not quite as good a stain as any of the foregoing, and superior to all except safranin in respect of permanency.

Mauvein and Rouge Fluorescent.

Solid Green.

Fuchsin (meaning the basic fuchsins, a series of Rosanilin salts having very similar reactions, and found in commerce under the names of Fuchsin, Anilin Red, Rubin, Roseïn, Magenta, Solferino, Corallin).—A good but somewhat weak stain. Good results are said to be obtained by substitution in the following manner (Graser, *Deutsche Zeit. f. Chirurgie*, xxvii, 1888, pp. 538—584; *Zeit. f. wiss. Mik.*, v, 3, 1888, p. 378). You either employ the colour as directed for methyl violet (*post*, § 285), or you stain for twelve to twenty-four hours in a dilute aqueous solution, wash out for a short time in alcohol, stain for a few minutes in aqueous solution of methylen blue, and dehydrate with alcohol. A double stain. Chromatin and nucleoli red, all the rest blue.

Ziehl's Carbolic Fuchsin has been recommended as superior to safranin by Schenk (*Ueber Conservirung von Kerntheilungsfiguren*, Bonn, 1890). I do not know where the original formula was published, and take this from *Zeit. f. wiss. Mik.* (vii, 1, 1890, p. 39).

The stain is made either by taking—

Fuchsin	1	gramme,
Acid. carbol. crist. . .	5	grammes,
Alcohol	10	,,
Aq. dest.	100	,,

or by saturating a 5 per cent. aqueous solution of carbolic acid with concentrated alcoholic solution of fuchsin (the saturation of the carbolic solution with fuchsin is made manifest by the formation of a metallic-looking pellicle on the surface of the liquid). The stain is washed out with alcohol followed by clove oil.

Orange, precise but weak.

Bismarck Brown is not very satisfactory with chromic objects. With alcohol objects it gives a good chromatin stain, but cannot be thoroughly removed from cytoplasm by any means yet discovered. It has this advantage, that being sufficiently resistent to alcohol it may be utilised for staining entire objects.

Kaiser (*Biblioth. Zool.*, H. 7, 1 Hälfte, 1891; *Zeit. f. wiss. Mik.*, viii, 3, 1891, p. 363) has obtained good results with Bismarck brown in the following way:—Stain for forty-eight hours, and at a temperature of 60° C., in saturated solution of Bismarck brown in 60 per cent. alcohol (the solution to be made in boiling alcohol), and wash out (until all is decoloured except the karyokinetic figures) in 60 per cent. alcohol, containing 2 per cent. hydrochloric acid or 3 per cent. acetic acid.

To these may be added—

METHYL VIOLET, perhaps best used according to the method of Resegotti given in § 278.

BENZOAZURIN has been lately recommended by MARTIN (see *Zeit. f. wiss. Mik.*, vi, 3, 1889, p. 193). Stain for an hour or so in dilute aqueous solution, and wash out with HCl alcohol.

Methylen blue may be used in the regressive way, and made to afford a chromatin stain.

Nigrosin has been recommended by ERRERA (*Proc.-Verb. Soc. Belge de Mic.*, 1881, p. 134). I have obtained some fine and vigorous chromatin stains with it in the regressive way. The stain resists alcohol well.

With **Toluidin Blue** I have had some superb stains of chromatin, unfortunately accompanied by a diffuse staining of cytoplasm.

MANN (*Zeit. f. wiss. Mik.*, xi, 4, 1894, p. 489) states that he has had good results by staining with it after eosin, thus obtaining a double stain, the eosin figuring as a plasma stain in the combination.

B. *Progressive Stains.*

282. As regards the progressive nuclear stains, the reader is reminded that many if not most of the anilins give a nuclear stain of greater or less purity if they are used in solutions acidified with acetic acid. Under the present heading, only those are mentioned which give in all respects, alike as regards precision and permanence, simplicity of manipulation and other qualities, a really valuable stain. The very existence of methyl green and Bismarck brown is a sufficient reason for being silent, in this connection, with regard to the rest.

283. Methyl Green.—This is the most common in commerce of the "anilin" greens. It appears to go by the synonyms of *Methylanilin green*, *Grünpulver*, *Vert Lumière*, *Lichtgrün*; these two last are in reality the names of another colour. When first studied by Calberla, in 1874 (*Morphol. Jahrb.*, iii, 1887, p. 625), it went by the name of *Vert en cristaux*. It is commonly met with in commerce under the name of more costly greens, especially under that of Iodine green. It is important not to confuse it with the latter, nor with Aldehyde green (*Vert d'Eusèbe*), nor with the phenylated rosanilins, *Paris green*, and *Vert d'Alcali*, or *Véridine*.

Methyl green is the chloromethylate of zinc and penta-methyl-rosalin-violet. It is obtained by the action of methyl chloride on methyl violet. The commercial dye frequently contains unconverted methyl violet as a consequence of defective purification. It is sometimes adulterated with anilin blue (soluble blue). It is also sometimes adulterated with a green bye-product of the manufacture,—the chloride of nona-methyl-para-leukanilin (see BENEDIKT and KNECHT's *Chemistry of the Coal-tar Colours*).

Methyl green is extremely sensitive to the action of alkalies. It is therefore important to use it only in acidified solutions, and to use only acid, or at least perfectly neutral fluids for washing and mounting.

This is an *extremely important histological reagent*. Its chief use is as a *chromatin* stain for *fresh* or *recently fixed tissues*. For this purpose it should be used in the form of a strong aqueous solution containing a little acetic acid (about 1 per cent. in general). *The solutions must always be acid*. You may wash out with water (best acidulated) and mount in some acid aqueous medium containing a little of the methyl green in solution. The mounting medium, if aqueous, *must be acidulated*.

Employed in this way, methyl green is a pure chromatin stain, in the sense of being a precise colour reagent for chromatin. For *in the nucleus* it stains nothing but the chromosomes, or chromatin elements: it does not stain plasmatic nucleoli, nor caryoplasm, nor achromatic filaments. *Outside* the nucleus it stains some kinds of cytoplasm and some kinds of formed material, especially glandular secretions (silk, for instance, and mucin). But the chromatin elements are invariably stained of a bright green (with the exception of the nucleïn of the head of some spermatozoa), whilst extra-nuclear structures are in general stained in tones of blue or violet.

Besides being a perfectly precise test for chromatin in the nucleus, methyl green has other advantages. Staining is *instantaneous;* over-staining never occurs. The solution is very penetrating, kills cells instantly without swelling or other change of form, and preserves their forms for at least some hours, so that it may be considered as a delicate fixative. It may be *combined without precipitating* with divers fixing or preserving agents. Osmic acid (0·1 to 1 per cent.) may be added to it, or it may be combined with solution of RIPART and PETIT (this, by the way, is an excellent medium for washing out in and mounting in).

Alcoholic solutions may also be used for staining. They also *should be acidulated with acetic acid.*

The stain does not keep easily. It is difficult to mount it satisfactorily in balsam, because the colour does not resist alcohol (unless this be sufficiently charged with the colour). The resistance of the colour to alcohol is, however (at all events if it be used in the Ehrlich-Biondi combination), considerably increased by treating the sections for a few minutes with tincture of iodine before staining (M. HEIDENHAIN). And SQUIRE declares that thorough washing with water before passing into the alcohol has the same effect.

Of preparations mounted with excess of colour in the usual aqueous media the most fortunate only survive for a few months. Dr. HENNEGUY, however, writes to me that it keeps well in BRUN's glucose medium.

Undoubtedly methyl green is one of the most valuable stains yet known. *It is the classical chromatin stain for fresh tissues.*

It was first pointed out, I believe, by Heschl (*Wiener med. Wochenschr.*, 2, 1879), that methyl green is a reagent for amyloid degeneration. His observations were confirmed by Curschmann (*Virchow's Arch.*, vol. lxxix, 1880, p. 556), who showed that it colours amyloid substance of an intense violet; but this (as pointed out by SQUIRE, *Methods and Formulæ*, &c., Churchill, 1892, p. 37) may be due to its containing methyl violet as an impurity.

The following paragraph, translated from the paper by Calberla above quoted, appeared inadvertently without comment in the first edition, and has since been repeated without comment in several places:

"He then found that 'the nuclei of subcutaneous connective tissue and those of vessels and nerve-sheaths stained rose-red, cells of the corium reddish white, and the cells of epidermis greenish blue to pure blue.'"

It is impossible at the present day to make out what can have been the origin of this astonishing statement. Methyl green certainly never under any circumstances stains nuclei red. I suggested in the last two editions that Calberla's observation should be taken to refer to the results of a double stain with methyl green and eosin, which is mentioned in the somewhat obscurely expressed passage from which the quotation is taken. PAUL MAYER however (*in litt.*, and see *Mitth. Zool. Stat. Neapel*, xii, 2, 1896, p. 311), has examined the passage in question, and is of opinion that it can hardly bear the interpretation I put upon it. He prefers to suppose that Calberla's colour contained methyl violet. That is quite a plausible supposition; but even then the nuclei could hardly have stained red, unless they were in a state of amyloid degeneration.

Calberla's misstatement is repeated, and made one of the grounds of an important theoretical deduction by Griesbach, in *Zeit. f. wiss. Mik.*, iii, 3, 1886, p. 365—an instructive example of the way in which history is written in some quarters.

284. Bismarck Brown (**Manchester Brown, Phenylen Brown, Vesuvin, La Phénicienne**).—A fairly pure nuclear stain that will work either with fresh tissues or with such as have been hardened in chromic acid.

The colour is not very easily soluble in water. You may boil it in water, and filter after a day or two (Weigert, in *Arch. f. mik. Anat.*, xv, 1878, p. 258). You may add a little acetic or osmic acid to the solution. Maysel (*ibid.*, xviii, 1880, pp. 237, 250) dissolves the colour in acetic acid (this solution does not give a permanent stain). Alcoholic solutions may also be used; or Calberla's glycerin-and-alcohol mixture, or dilute glycerin (say of 40 per cent. to 50 per cent.) may very advantageously be employed.

The watery solutions must be frequently filtered. The addition to them of carbolic acid has lately been recommended (vide *Journ. Roy. Mic. Soc.*, 1886, p. 908). Bismarck brown stains rapidly, but never overstains. The stain is permanent both in balsam and in glycerin.

The chief use of this colour is for staining objects *in toto*; but it may also be employed for staining sections by the regressive method.

As has been noted above (§ 213), Bismarck brown has the property of imparting a *quasi*-stain to certain cellular elements during life (for this purpose it is necessary to see that the colour employed be pure *and neutral*).

285. Methyl Violet (Methylanilin = anilin-violet = Paris violet = inchiostro di Leonardi).—The following process has been recommended by Orth (*Amer. Mon. Micr. Journ.*, i, 1880, p. 143; *Journ. Roy. Mic. Soc.*, N.S., i, 1881, p. 137):—Sections are to be soaked in water, and then brought into the following solution:

Anilin violet	1 part.
Acetic acid	300 parts.

Mount, without washing out, but simply draining, in acetate of potash (acetate 2 parts, water 1 part).

The stain will probably fade within a year or two.

This process does not appear to be of more than very limited applicability. The following, however, due to Graser (*Deutsche Zeit. f. Chirurgie*, xxvii, 1888, pp. 538—584; *Zeit. f. wiss. Mik.*, v, 3, 1888, p. 378), may be very generally useful.

Sections are stained for from twelve to twenty-four hours in a (presumably aqueous) solution so dilute that at the end of that time the sections will have taken up all the colour from the liquid. They are then washed out for a short time in acidulated alcohol, and then in pure alcohol (followed presumably by clearing and mounting in balsam). Schiefferdecker, whose account is here quoted, says that the results, as regards nuclear figures, are even finer than with safranin. The method is applicable to objects fixed in "Flemming."

A useful stain for fresh tissues is also obtained by using dilute acetic acid in the manner recommended above (by Ehrlich) for Dahlia, (§ 281).

Amyloid matter appears red in preparations stained with methyl violet. This appears to be an optical effect (see the curious experiments of Capparelli, in *Archivio per le scienze mediche*, iii, No. 21, p. 1; but see also Paul Mayer, in *Mitth. Zool. Stat. Neapel*, xii, 2, 1896, p. 328, who has not been able to obtain Capparelli's results).

REESE LIBRARY
OF THE
UNIVERSITY
OF CALIFORNIA

CHAPTER XVII.

METHYLEN BLUE.

286. Methylen blue is the chloride or the zinc chloride double salt of tetramethylthionin, and is a "basic" colour in Ehrlich's sense. It appears that some persons have confounded it with methyl blue, which is either a basic colour derived from diphenylamine blue, or an acid colour obtained by the sulphonation of diphenylamine blue; neither of which colours has histologically any resemblance to methylen blue.

Commercial methylen blue sometimes contains as an impurity a small quantity of methylen red. This is not an undesirable factor; on the contrary, it sometimes affords differentiations of elements of tissues or of cells that cannot be produced by any other means. Samples containing methylen red are not so common now as formerly. The colour in this state is known as *polychromatic methylen blue,* and is employed for staining certain cell-granules. It can be obtained from Grübler and Co.

The colour to be employed for *intra-vitam nerve staining* should, on the contrary, be as pure as possible. Apáthy has a note in *Zeit. f. wiss. Mik.*, ix, 4, 1893, p. 466, to the effect that the best methylen blue for impregnation—in fact, the only one that will give *exactly* the results described by him (see § 292)—is that obtained from E. Merck, of Darmstadt, and quoted in his price list as "*medicinisches* Methylenblau," and described on the label as "Anilin-blau, Methylen, chemisch rein und chlorzinkfrei." It is therefore highly desirable, when ordering methylen blue, to specify for what purpose it is required.

287. The Uses of Methylen Blue.—This colour seems to be in a fair way to become one of the most important reagents in the histological laboratory. Its importance as a stain for micro-organisms in tissues is well known to all pathologists.

As a histological reagent it is used for sections of hardened central nervous tissue, in which it gives a specific stain of medullated nerves (*post,* Part II). It is a valuable specific reagent for plasma-cells (for which see also Part II). It possesses the property of washing out the stain of certain other anilins, with which it gives valuable double stains (next chapter). It stains a large number of tissues *intra vitam,* with little or no interference with their vital functions. And last, not least, it can be made to furnish stains of nerve-tissue, intercellular cement substances, lymph spaces, and the like, that are essentially identical with those furnished by a successful impregnation with gold or silver. The results are quite equal in most cases to those of gold or silver impregnation, and are obtained with far greater ease and certainty. I call especial attention to these processes, which seem to be effecting a revolution in histological technique.

288. Staining *in toto* during Life.—Small and permeable aquatic organisms may be stained during life by adding to the water in which they are confined enough methylen blue to give a scarcely perceptible or at least very light tint to the water. If transparent organisms be taken, they may be examined alive without further manipulation at any desired moment. Microscopically examined, they will be found after a time to be partially stained—that is, it will be found that certain tissues have taken up the colour, others remaining colourless. If now you put back the animals into the dilute solution and wait, you will find on examination after a sufficient lapse of time that further groups of tissues have become stained. Thus it was found by EHRLICH (*Abh. k. Akad. Wiss. Berlin,* February 25, 1885), to whom the principle of the methods under consideration is due, that on injection of the colour into living animals axis-cylinders of *sensory* nerves stain, whilst *motor* nerves remain colourless. [The motor nerves, however, will also stain, though later than the sensory nerves.] It might be supposed that by continuing the staining for a sufficient time, a point would be arrived at at which all the tissues would be found to be stained. This, however, is not the case. It is always found that the stained tissues only keep the colour that they have taken up for a short time after they have attained the maximum degree of coloration of which they are susceptible; as soon as that point is attained they begin to discharge the colour even more quickly than they took it up. And it is very often found that the elements which have stained first will have lost much or all of their colour by the time that those which stain later have attained their maximum coloration. It may even happen, as I have observed, that the whole of the stainable tissues of an animal may run through the total gamut of coloration and decoloration until the animal has become as colourless as when first put into the tinted water, and that without any apparent change in its vital activities. The stain, therefore, that has thus been produced and lost is not a true histological stain, but a *quasi*-stain (see § 213).

It follows that a total stain of all the tissues of an organism can hardly be obtained under these conditions, but that a specific stain of one group or another of elements may be obtained in one of two ways. If the tissue to be studied be one that stains earlier than the others, it may be studied during life at the period at which it alone has attained the desired intensity of coloration, and the remaining tissues are not yet coloured at all, or not coloured enough to be an obstacle to observation. If it be one that stains later than the others, it may be studied during life at the period at which the earlier stained elements have already passed their point of maximum coloration and have become sufficiently decoloured not to be an obstacle to the observation of the later stained ones; the latter being either at the point of maximum coloration or at a point of desired intensity either earlier or later than the maximum. Or the observer may fix the stain in either of these stages and preserve it for leisurely study by means of one of the processes given below under the heading "*Preservation of the Preparations.*"

The proper strength of the very dilute solutions to be employed in the manner here considered must be made out by experiment for each object. I think the tint is practically a sufficient guide, but it may be stated that when in doubt a strength of 1 : 100,000 may be taken, and increased or diminished as occasion may seem to require. ZOJA (*Rendic. R. Ist. Lombardo*, xxv, 1892; *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 208) finds that for Hydra the right strength is from 1 : 20,000 to 1 : 10,000.

The stain is somewhat capricious. It is not possible to predict without trial which tissues will stain first in any organism. It is to be remarked that the stain penetrates very badly, which is perhaps the chief cause of its seeming capriciousness, and, I take it, the chief determining condition of the order in which tissues stain. Gland-cells generally stain early; then, in no definable order, other epithelium-cells, fat-cells, plasma-cells, "Mastzellen," blood and lymph corpuscles, elastic fibres, smooth muscle, striated muscle. There are other elements that stain in the living state, but *not* when the staining is performed by *simple immersion of intact animals* in a dilute staining solution in the manner we are considering. Chief amongst these are *nerve-fibres* and *ganglion-cells, which remain unstained in the intact organism,*—most likely, so far as I can see, for the simple reason that the stain is not able to penetrate them.

289. Staining Nervous Tissue during Life.—As was stated in the last section, it was made out by EHRLICH that by injecting a solution of methylen blue into the vessels or tissues of living animals there may be obtained a specific stain of axis-cylinders of sensory nerves. He held, and it has long been held by the majority of observers, that the stain so obtained is a product of a *vital* reaction of the tissues, and that it cannot be obtained with dead material. From the point of view maintained in § 213, the contrary would be the real state of the case. The stain is, of course, an *intra-vitam* phenomenon in so far as it takes place during the life of the organism; but I hold that the tissue itself does not take on the stain till it is dead or moribund.

As said before, it was formerly held that the reaction could not be obtained with dead material. DOGIEL, however (*Arch. f. mik. Anat.*, xxxv, 1890, pp. 305 *et seq.*), found that muscle-nerves of limbs of the frog could be stained as much as from three to eight days after the limbs had been removed from the animal. He concludes, indeed, that the reaction shows that the nerves were still living at that time. But it seems more natural to conclude with APÁTHY (*Zeit. f. wiss. Mik.*, ix, 1, 1892, pp. 15 *et seqq.*) that nerve-tissue can be stained after life has ceased.

APÁTHY has directly experimented on this point, and sums up the necessary conditions as follows:—The tissue need not be living, but must be fresh; nothing must have been extracted from it chemically, and its natural state must not have been essentially changed by physical means. For example, the tissue must not have been treated with even dilute glycerin, nor with alcohol, though a treatment for a short time with physiological salt solution is not very hurtful; it must not have been coagulated by heat.

Another common belief concerning the methylen-blue nerve reaction is that the presence of oxygen is necessary to the reaction. It is, therefore, the usual practice to dissect out the organ to be investigated after having exposed it to the action of methylen blue by injection or immersion, and leave it for some time exposed to the air. APÁTHY has also investigated this point, and finds (loc. cit., p. 25) that the practice is in some cases correct, but the belief erroneous. His point of view is that the stain is a *regressive* one. It has been explained above that shortly after a tissue has attained the maximum degree of coloration of which it is susceptible it begins to give up its colour again to the surrounding liquid. The larger the volume of liquid with which the tissue is surrounded, the faster will this washing-out process go on; and in order that it may not go on with excessive rapidity, washing out the stain from the nerve-fibres as well as from the earlier stained elements (which alone it is desired to wash out, so as to leave a differentiated specific stain of nervous elements), it is in many cases desirable to have the process go on in presence of as little liquid as possible. Another consideration that justifies the practice is that by exposure to air the preparations take up a trace of *ammonia* (derived from the ammonium carbonate of the air); and Apáthy has experimentally established that this is an important factor in the sharpness of the stain. Oxygen has, according to him, nothing to do with it.

290. Staining Nerve-tissue by Injection or Immersion.—The practice of the earlier workers at this subject was (following Ehrlich) to inject methylen blue into the vascular system or body-cavity of a living animal, wait a sufficient time for it to take effect on the organ to be stained, then remove the organ for further preparation and study. And there appears to have been a belief with some workers that it was an essential or at least a desirable condition to the production of the stain that it should have been brought about by injection of the colouring matter into the entire animal. It is now known that this is generally immaterial, and that the reaction can equally well be obtained by removing the organ and subjecting it to a bath of the colouring matter in the usual way. So that treatment with the colour by means of injection or by means of immersion in a solution may be taken to be, in general, a matter of convenience only. But it would also appear that in some cases the procedure by injection is preferable, if not necessary. Bürger (*Mitth. Zool. Stat. Neapel*, x, 1891, p. 206) found that for Nemertians it was necessary to inject the animals thoroughly, and leave them to dry, before the stain could be obtained.

291. The Solutions employed.—The solutions used *for injection* are generally made in salt solution (physiological, or a little weaker); those used for staining by immersion are made either in salt solution or other "indifferent" liquid, or in pure water. Very various strengths of solution have been employed. The earlier workers generally took concentrated solutions. Thus Arnstein (*Anat. Anz.*, 1887, p. 125) injected 1 c.c. of saturated solution into the vena cutanea magna of frogs, and removed the organ to be investigated after the lapse of an hour. Biedermann (*Sitzb. d. k. Akad. Wiss. Wien, Math. Nat. Cl.*, 1888, p. 8) injected 0·5 to 1 c.c. of a nearly saturated solution in 0·6 per cent. salt solution into the thorax of crayfishes, and left the animals for from two to four hours before killing them. Mayer (*Zeit. f. wiss. Mik.*, vi, 4, 1889, p. 423) took a strength of 1 : 300 or 400 of 0·5 per cent. salt solution. This can be introduced into the system either by means of a syringe or other injecting apparatus, or by auto-injection through the heart. Even rabbits support this operation if artificial respiration be maintained. The solutions of Retzius are of the same strength. But the tendency of more recent practice is decidedly towards the employment of weaker solutions. Apáthy (*Zeit. f. wiss. Mik.*, ix, 1, 1892, pp. 25, 26, *et seq.*) finds that it is not only superfluous, but positively disadvantageous, to take solutions stronger than 1 : 1000.

For staining *by immersion* similar solutions to those used for injecting may be employed, but they should, if anything, be still weaker. Dogiel (*Arch. f. mik. Anat.*, xxxv, 1890, p. 305; *Zeit. f. wiss. Mik.*, vii, 4, 1891, p. 509) places objects in a few drops of aqueous or vitreous humour, to which are added two or three drops of a $\frac{1}{16}$ to $\frac{1}{15}$ per cent. solution of methylen blue in physiological salt solution, and exposes them therein to the air. In thin pieces of tissue the stain begins to take effect in five or ten minutes, and attains its maximum in from fifteen to twenty minutes. For thicker specimens—retina, for instance—several hours may be necessary, the preparation being kept just moist by occasional treatment with a drop or two of indifferent liquid or methylen-blue solution, added by turns. The reaction is quickened by putting the preparations into a stove kept at 30° to 35° C. Rouget (*Compt. Rend.*, 1893, p. 802) found it useful to modify the procedure of Dogiel by employing a 00·5 per cent. solution in 0·6 per cent. salt solution (for muscles of Batrachia). Allen (*Quart. Journ. Mic. Sci.*, 1894, pp. 461, 483) takes for embryos of the lobster a solution of 0·1 per cent. in salt solution, and dilutes it with 15 to 20 volumes of sea water.

292. Apáthy's **Methods.**—As a good example of this kind of work, I subjoin a short account of the procedure recommended by Apáthy (*Zeit. f. wiss. Mik.*, ix, 1, 1892, p. 15) for *Hirudinea.* A portion of the ventral cord is exposed, and if it be considered desirable dissected out, but the sinus and pigmented connective tissue around it had better not be removed till the staining and fixation are completed. If, however, it be desired to stain as many ganglion-cells as possible, as well as fibres, the lateral nerves, as well as the connectives, should be cut through near a ganglion. The preparation is then treated with the stain. This is, for the demonstration chiefly of fibres in *Hirudo* and *Pontobdella*, either a 1 : 1000 solution in 0·5 to 0·75 per cent. salt solution, allowed to act for ten minutes; or a 1 : 10,000 solution allowed to act for an hour to an hour and a half; or a 1 : 100,000 solution allowed to act for three hours (*Lumbricus* requires twice these times; *Astacus* and *Unio* require three times; medullated nerves of Vertebrates four times). For the demonstration of ganglion-cells the stain is allowed to act three or four times as long (the connective and lateral nerves having been cut as directed above).

The staining having been accomplished, the preparations from the 1 : 1000 solution are washed in salt solution for an hour; those from the 1 : 10,000 solution for a quarter of an hour; those from the 1 : 100,000 solution need not be washed at all. They are then treated with one of the ammoniacal fixing and differentiating liquids described below in the next section. This is done by pouring the liquid over them, and leaving them in it *without moving them about in it* for at least an hour, and by preference in the dark. The further treatment is as described in the next section.

The object of the ammonia in these liquids is to *differentiate* the stain—to produce an artificial "secondary differentiation." It acts by washing out the absorbed colour from certain elements, others resisting its action longer, much as HCl alcohol washes out a borax-carmine stain. In this case the elements that are washed out are the protoplasmic parts of nerve-fibres, and their "interfibrillar" and "perifibrillar" substance, the "primitive fibrils" still retaining the colour strongly. It is of theoretical interest to remark that according to Apáthy the coloration thus obtained is a true *stain* of the "primitive fibrils," not an *impregnation.* The "primitive fibrils" are sharply stained of a violet-blue, showing no granular precipitate, and the "interfibrillar" and "perifibrillar" substance, as well as nuclei, are either colourless or very lightly stained. The usual methods, on the other hand, give an "inverse" reaction, the "primitive fibrils" remaining colourless, whilst the interfibrillar substance and protoplasm of the nerve-fibres are *impregnated* with a finely granular greenish-black or violet precipitate, and the nuclei are usually stained.

293. Preservation of the Preparations.—There are considerable difficulties in the way of obtaining permanent preparations of methylen blue stains, as the stain is so very unstable that, as above explained, it begins to discharge after a short time, even in the living and not yet totally impregnated tissue. The colour may, however, be fixed, and more or less permanent preparations be made by one or other of the following methods:

Dogiel (*Arch. f. mik. Anat.*, xxxiii, 4, 1889, pp. 440 *et seq.*), following Arnstein (*Anat. Anzeig.*, 1887, p. 551), brings the preparations, in order to fix the colour, into saturated aqueous solution of picrate of ammonia, in which they are allowed to remain for half an hour or more, and are then removed, washed in fresh picrate of ammonia solution, and studied in dilute glycerin, or mounted permanently in glycerin saturated with picrate of ammonia. More recently (*Zeit. f. wiss. Mik.*, viii, 1, 1891, p. 15) he has recommended an increased duration of the picrate of ammonia bath up to eighteen or twenty-four hours, and mounting, without washing out, in chemically pure glycerin, free from acid. There is a defect in this process, namely, that picrate of ammonia has a very injurious action of a macerating nature on some tissues. This may, however, be avoided by adding to the fixing-bath 1 to 2 per cent. of a 1 per cent. osmic acid solution. (If it be desired to harden the tissues so that sections may be cut, the proportion of osmium solution should be increased fourfold.)

S. Mayer (*Zeit. f. wiss. Mik.*, vi, 4, 1889, p. 422) preferred a mixture of equal parts of glycerin and saturated picrate of ammonia solution, which served to fix the colour and mount the preparations in. This was also in principle the method followed by Retzius (*Intern. Monatsschr. Anat. u. Phys.*, Bd. vii, H. 8, 1890). Dogiel, after careful study, quite refuses to admit that this is in any way an improvement.

Other workers have employed saturated solution of iodine in iodide of potassium (so Arnstein) or picro-carmine (so Feist, *Arch. f. Anat. u. Entwickel.*, 1890, p. 116; cf. *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 231), the latter having the advantage of preserving the true blue of the stain if it be not allowed to act too long, and the preparation be mounted in pure glycerin.

Picric acid has been used by Lavdowsky, but this too, after careful study, is rejected by Dogiel.

Apáthy (*Zeit. f. wiss. Mik.*, ix, 1, 1892, p. 30) has found, as stated above, that free ammonia is a capital factor in the differentiation of the stain. He brings preparations (after washing in salt solution if the staining have been performed with a strong methylen blue solution, or without washing if it have been done with a very dilute solution) either into a concentrated aqueous solution of picrate of ammonia *free from picric acid*, and containing five drops of concentrated ammonia for every 100 c.c.; or, which is generally preferable, into a 1 to 2 per cent. freshly prepared solution of neutral carbonate of ammonia, saturated with picrate. They remain in either of these solutions, preferably in the dark, for *at least an hour*. They are then brought into a *small* quantity of saturated solution of picrate of ammonia in 50 per cent. glycerin, where they remain until thoroughly saturated. They are then removed into a saturated solution of the picrate in a mixture of 2 parts 50 per cent. glycerin, 1 part cold saturated sugar solution, and 1 part similarly prepared gum-arabic solution. When thoroughly penetrated with this they are removed and mounted in the following gum-syrup medium (l. c., p. 37):

Picked gum-arabic . . .	50 grms.
Cane-sugar (not candied) . .	50 ,,
Distilled water	50 ,,

Dissolve over a water-bath and add 0·05 grm. thymol. This mounting medium sets quickly and as hard as balsam, so that no cementing of the mounts is necessary. Farrant's medium (with omission of the arsenious acid) will also do. In neither case should either ammonium picrate or methylen blue be added to the medium.

Preparations preserved by these methods (I do not know whether it is the case with preparations preserved by Parker's or Bethe's method, next section) are extremely sensitive to the influence of light. Diffused daylight is less injurious to them than the light concentrated on them by the illuminating apparatus of the microscope during observation. Apáthy finds that lamplight is particularly injurious, especially the intense lamplight used with high powers; which he attributes partly to the yellow rays, partly to the heat.

294. Methods for Sections.—None of the preceding methods can be said to be anything like perfectly satisfactory. They do not give preparations that will resist the operations necessary for imbedding in paraffin. The stain is generally not preserved in its true blue colour, but turns to a grey, varying in tone from reddish brown to bluish or greenish black. The preparations seldom keep even in that state for more than a very few months, and it is not satisfactory to be obliged to mount preparations only in aqueous media. A strong solution of platinum chloride is said to give a fixation that will resist the treatment necessary for imbedding either in celloidin or paraffin (see FEIST, *Arch. f. Anat. und Entw.*, 1890, p. 116; *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 231); but the precipitate it gives is a flocculent one, and the preparations are not very satisfactory.

The method of PARKER (*Zool. Anzeig.*, No. 403, 1892, p. 375) is therefore a most welcome step forwards. The stain is fixed (1) (in the form of a finely grained purplish precipitate) by cold concentrated aqueous solution of corrosive sublimate. The preparations are dehydrated (2) in a solution composed of 1 grm. of sublimate and 5 c.c. of *methylal* (pure methylal washes out the stain to a certain extent). The methylal solution is now removed by means of a mixture (3) of two parts xylol, one part pure methylal, and one part of the dehydrating mixture (2). After a short time the preparations are placed in (4) a considerable quantity of xylol. Here they should remain till all the methylal is replaced by xylol and the corrosive sublimate completely washed out. This will take some four or five days, as sublimate is very little soluble in xylol. Mount in balsam, or if sections be desired, imbed in paraffin in the usual way. Sections should be fixed to the slide with Schällibaum's collodion, and not with Mayer's albumen, which discharges the colour. The preparations will keep for several weeks, but the finer details are likely to fade after a month.

The times required for a ganglion of the ventral chain of a crayfish are for (1) ten minutes, (2) fifteen minutes, (3) ten minutes, (4) four or five days.

Feist, in the paper quoted above, recommends Joliet's gum-glycerin imbedding method; but in most cases the method of Parker will doubtless be preferable.

The method of Bethe (*Arch. f. mik. Anat.*, xliv, 1894, p. 585) is as follows:—A solution is made of—

Molybdate of ammonium	1 grm.
Distilled water	10 grms.
Peroxide of hydrogen	1 grm.

On adding the peroxide a yellow colour is produced. A drop of hydrochloric acid is added (white precipitate of molybdic acid which dissolves on agitation). After staining and rinsing in salt solution the preparations are put into the molybdic solution. The solution ought not to be more than eight days old, and it is well to use it cooled to zero. The preparations are left in it for two or three hours if they are small, or four or five hours if they are large (of a centimetre in size). They are then washed for from half an hour to two hours in water, dehydrated in alcohol (which it is well to use cooled to zero), and cleared in clove oil or, better, in xylol. They may then be imbedded either in paraffin or celloidin in the usual way.

This is for tissues of Vertebrates. For Invertebrates Bethe takes one grm. of molybdate, 10 c.c. of water, and 0·5 c.c. of peroxide.

295. Methylen Blue Impregnation of Epithelia, Lymph-spaces, &c. (Dogiel, *Arch. f. mik. Anat.*, xxxiii, 4, 1889, p. 440, *et seq.*).—Suitable pieces of tissue (thin membranes by preference) are brought fresh into a 4 per cent. solution of methylen blue in physiological salt solution. After a few minutes therein they are brought into saturated solution of picrate of ammonia, soaked therein for half an hour or more, then washed in fresh picrate of ammonia solution, and examined in dilute glycerin.

If it be wished only to demonstrate the outlines of endothelium cells, the bath in the stain should be a short one, not longer than ten minutes in general; whilst if it be desired to obtain an impregnation of ground-substance of tissue so as to have a negative image of juice-canals or other spaces, the staining should be prolonged to fifteen or thirty minutes, and it is advisable to remove the endothelial covering of the objects operated on before putting them into the stain.

If it be desired to preserve the preparations permanently, they had better be mounted in glycerin saturated with picrate of ammonia. (For an improvement in the method of preservation given in a later paper see *supra*, § 293.)

The effect is practically identical (except as regards the colour) with that of a negative impregnation with silver nitrate.

296. Mayer's (S.) **Impregnation Methods** (*Zeit. f. wiss. Mik.*, vi, 4, 1889, p. 422).—Mayer's experiments were made contemporaneously with those of Dogiel, cover much the same ground, and give the same results. Mayer stained tissues for about ten minutes in a 1 : 300 or 400 solution of methylen blue in 0·5 per cent. salt solution, rinsed in salt solution, and put up in the glycerin-picrate of ammonia mixture given above, § 293. He finds it may be stated that by this method *all the essential results of a nitrate of silver impregnation may be produced by means of methylen blue.* The images are either positive or negative. In stratified epithelia and in endothelia (for instance, in the testicular canals of the rat, in the vascular system, in smooth muscle) cement-substance is stained. In the cornea, ground-substance is stained, giving a negative image of the corneal corpuscles; and in like manner a negative image is sometimes obtained of the intra-sarcolemmar nerve-endings in striated muscle of frogs and rats. But methylen blue sometimes, as does sometimes silver nitrate, gives positive images of the cornea. If the stain be brought about by injection of the colour into the vascular system, the positive impregnation is the more frequent; whilst if it have been brought about by the immersion of the cornea, a negative image is more frequently obtained. In medullated nerves, striking images of the cruciform figures in the constrictions of Ranvier are obtained, just as with silver nitrate.

To sum up, almost any reaction that can be obtained with chloride of gold, or with nitrate of silver, can be obtained with methylen blue, and with much greater ease and certainty.

297. Other Uses of Methylen Blue.—Methylen blue is also used, chiefly in conjunction with other colouring matters, for staining fixed and hardened tissues, especially preparations of central nervous system. These uses will be described in their proper places.

CHAPTER XVIII.

THE COAL-TAR PLASMA STAINS.

298. As to Plasma Stains.—By a plasma stain is generally meant, rather vaguely, one that stains the extra-nuclear parts of cells and the formed material of tissues, or one of these. To be precise, the class ought to be subdivided, and we ought to speak of cytoplasm stains, granule stains, ground-substance stains, or the like. But the vague general sense of the term will be sufficient for the purposes of the present chapter.

In the earlier editions of this work I fought against the needless employment of plasma stains. But it must now be admitted that with the increasing differentiation of the problems of histology the employment of plasma stains has become in very many cases a necessity. Histology no longer stops short at the study of the groupings of cells; it now inquires into the inner differentiations of cells and of intercellular substances.

Good plasma stains are therefore much wanted. Unfortunately such a thing can hardly be said to exist; for it is not enough to require of a plasma stain that it should stain extra-nuclear material. It is also desirable that it should do so in as selective a way as possible. Now all plasma stains are more or less diffuse stains. Many exhibit considerable selectivity, but it is by no means always easy to get them to display the particular selectivity that is desired. Those that do not display it are of little use, or of none at all. I have therefore suppressed a large number of formulæ which appear to me to have little or no scientific value, and have not thought it worth while to cumber the text with references to the sources where they may be found. Most of them, if required, will be found quoted in the previous editions.

299. Picric Acid.—I follow Flemming (*Zeit. f. wiss. Mik.*, i, 1884, p. 360) in pointing out that picric acid is the most generally useful of all secondary stains. It gives useful plasma stains with most of the nuclear stains, and particularly with carmine and hæmatoxylin. It may be used with the most delicate of these stains, even the delicate coloration of alum-carmine being in no wise injured by it. The *modus operandi* is as simple as possible: it consists merely in adding picric acid to the alcohols employed for dehydrating the objects after staining with a nuclear stain.

It should be borne in mind that picric acid has considerable power of washing out other anilin stains; and that *in combination with hydrochloric acid it very greatly enhances* the power with which this acid washes out carmine stains. It should therefore not be added to the acidulated alcohol taken for differentiating borax-carmine stains, or the like, but only to the neutral alcohol used afterwards. It does not otherwise affect any of the usual stains, and may be most highly recommended as a useful though frequently inelegant stain. It has the great quality, shared by very few plasma stains, that it can be used for staining *entire objects.* And as it is extremely penetrating, it is very much indicated for the preparation of such objects as small Arthropods or Nematodes, mounted whole.

It can in some cases be employed by dissolving it in the solution of another dye (see Picro-carmine, Legal's alum-carmine, § 228, &c.).

300. Orange G.—This is the benzenazo-beta-naphthol-disulphonate of soda (to be obtained from Grübler and Co., and not to be confounded with about a dozen other colours that are on the market under the name of "Orange," with or without a suffix). As indicated by its chemical description, this is an "acid" colour in Ehrlich's sense; and it is also an acid colour in the usual sense, for its solution in water has an acid reaction. The solutions do not keep well, throwing down very quickly a pulverulent deposit.

I think this is one of the most precise cytoplasm stains that I have met with. But it is not very useful, because the stain is a very pale one. But it may be found useful as a secondary stain to come after a hæmatein stain or other blue or red stain. I use a saturated solution in water, and allow it to act for five or ten minutes (sections only). There is no possibility of over-staining taking place, but some other anilin colours may be washed out by it. The chief use of this stain is in combination with other anilins, as in Flemming's Orange Method, and the Ehrlich-Biondi-Heidenhain mixture, which see (§ 306).

301. FLEMMING'S **Orange Method** (*Arch. f. mik. Anat.*, xxxvii, 1891, p. 249; *ibid.*, p. 685; *Zeit. f. wiss. Mik.*, viii, 2, 1891, p. 223, and viii, 3, p. 343).—This is a substitution method. You stain (for as much as two or three days, or even weeks if you want a very powerful stain) in strong alcoholic safranin solution diluted with anilin water (§ 278); rinse in distilled water; differentiate in absolute alcohol, containing at most 0·1 per cent. of hydrochloric acid, until hardly any more colour comes away; stain for one to three hours in gentian (either a simple very strong aqueous solution, or, if you prefer, you may stain by the method of Gram, § 279); wash for a short time in distilled water; treat with concentrated, or at least fairly strong, aqueous solution of orange, which in virtue of its acid properties washes out most of the gentian. After at most a few minutes, whilst pale violet clouds are still being given off from the sections on agitation, bring them into absolute alcohol until hardly any more colour comes away, clear in clove or bergamot oil, and mount in damar or balsam before the last pale clouds of colour have ceased to come away (the orange should be the *orange G*, introduced into commerce by *Meister, Lucius*, and *Brüning*, of Höchst, and also prepared by the *Actiengesellschaft für Anilinfabrication*, Berlin; it may be obtained from GRÜBLER, § 216).

This is not a triple stain in the sense of giving three different colours in the result; the orange does not act as a separate stain, but as an agent for the differentiation of the gentian stain. Chromatin and nuclei are purple-red; achromatic spindle-fibrils grey to grey-brown, or, in very favorable cases, violet, and are very clearly brought out; attractive spheres, centrosomes, polar corpuscles, and "Zwischenkörper" reddish in light stains, brown-violet to black-brown in strong ones.

N.B.—The description of the process given by FLEMMING as above presumably applies to the case of *loose* sections. Working with sections *fixed to the slide,* I find that it does not apply quite exactly. An important difference is that I do not find that the orange washes out any of the gentian; at all events, no perceptible clouds of colour come away. I have found it well to prolong the treatment with the orange to at least a quarter of an hour. The duration of the staining bath in the gentian, as given by Flemming, seems to me much too long for any objects; half an hour seems quite enough.

I consider this a very fine stain when successful. The results are as described by Flemming. But it is very capricious, one can never be certain of success. For this reason, and for others, I prefer for most objects the safranin and Kernschwarz stain described in the next chapter.

302. REINKE'S **Modification of** FLEMMING'S **Orange Method** (*Arch. f. mik. Anat.,* xliv, 2, 1894, p. 262).—Sections of material fixed in liquid of HERMANN are put for twenty-four hours into a concentrated solution of potassium sulphite. They are washed with water and stained for an hour or two in safranin. They are then well washed with water, and stained for twenty-four hours in a "neutral" mixture of gentian and orange, prepared as follows.

To a concentrated aqueous solution of gentian violet are added "a few drops" of a like solution of orange G. The solution precipitates in part, owing to the formation of an imperfectly soluble "neutral" colour; but becomes almost clear again if an excess of water be added. A drop of the mixture placed on blotting-paper should form a violet or brown spot with a narrow orange border. The solution is not to be filtered, but the sections are to be stained in it as it is (it is impossible to make out whether Reinke means the undiluted mixture, or the mixture made almost clear by addition of water). It is said that the "neutral" solution may be preserved for future use by adding to it one third of alcohol. After staining, you differentiate rapidly with alcohol, and clear with clove oil.

I have tried this process, and obtained exactly the same final results as with Flemming's process. I have not sufficient experience of it to be able to say whether it is any less capricious than Flemming's process.

303. **Metanil Yellow** (**Metanilgelb**). See GRIESBACH (*Zeit. f. wiss. Mik.*, iv, 4, 1887, p. 448; see also *Journ. Roy. Mic. Soc.*, 1889, p. 464). It is said to have a certain affinity for various elements belonging to the group of the connective tissues.

304. **Säuregelb (Echtgelb), Tropæolin O., Crocein, Gold Orange,** (see GRIESBACH, *Arch. f. mik. Anat.*, xxii, p. 132).

305. **Säurefuchsin (Acid Fuchsin, Fuchsin S, Acid Rubin, Rubin S, Säurerubin, Acid Magenta, Magenta S)**.—The denomination "**Patentsäurerubin**" attributed to this colour by KULTSCHIZKY (*Anat. Anz.*, viii, 1893, p. 357) is erroneous; the proper name of the colour used by Kultschizky is "**Rubin S**, rein pat.," see *Ergebnisse der Anatomie*, iii, 1893, pp. 18, 19). The chemical description of this acid colour has been given (§ 268): it is important not to confound it with basic fuchsin, as seems to have been done by some writers.

This is one of the best cytoplasm stains that I know of. I use a 0·5 per cent. solution in water, and allow it to act on sections for about five minutes. But it is a matter of chance whether one hits off exactly the desired effect in this way. The chief use of Säurefuchsin is as an ingredient in the EHRLICH-BIONDI-HEIDENHAIN stain, and EHRLICH'S Triacid, which see. It is also used as a specific stain for nerve tissue (see "Neurological Methods" in Part II).

306. **The** EHRLICH-BIONDI **Mixture** (or EHRLICH-BIONDI-HEIDENHAIN **Mixture**) (*Pflüger's Arch.*, xlii, 1888, p. 1; *Zeit. f. wiss. Mik.*, v, 4, 1888, p. 520).—This well-known stain is somewhat troublesome to prepare. It may be obtained ready made from GRÜBLER and Co.

The receipt is as follows:—To 100 c.c. saturated aqueous solution of Orange add with continual agitation 20 c.c. saturated aqueous solution of Säurefuchsin (Acid Fuchsin) and 50 c.c. of a like solution of Methyl Green.

(According to Krause, 100 parts of water will dissolve about 20 of Säurefuchsin [Rubin S.], 8 of Orange G, and 8 of Methyl Green.) The solutions must be *absolutely saturated*, which only happens after several days.

Dilute the mixture with 60 to 100 volumes of water. The dilute solution ought to redden if acetic acid be added to it; and if a drop be placed on blotting-paper it should form a spot bluish green in the centre, orange at the periphery. If the orange zone is surrounded by a broader red zone, the mixture contains too much fuchsin.

According to M. Heidenhain's instructions ("Ueber Kern u. Protoplasma," in *Festschr. Herrn. Geb. A. v. Kölliker gewidm.*, &c., 1892, p. 115; see *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 202) the orange to be used should be "Orange G;" the Acid Fuchsin or Säurefuchsin should be "Rubin S" ("Rubin" is a synonym of Fuchsin); and the Methyl Green should be "Methylgrün OO." And it is *absolutely necessary* that these ingredients be those prepared under those names by the *Actienfabrik für Anilinfabrication in Berlin.*

The strong solutions directed to be taken readily precipitate on being mixed. To avoid this it is recommended by Squire (*Methods and Formulæ*, &c., p. 37) to dilute them before mixing.

Stain sections (N.B., *sections only*) for six to twenty-four hours. Dehydrate with alcohol, clear with xylol, and mount in xylol balsam.

In the intention of the observers who have elaborated this stain, it is a *progressive* stain, and *not* a regressive one. It does not require any differentiation, and the sections should be got through the alcohol into xylol as quickly as possible in order to avoid any extraction of the colour. The great point of difficulty in working with this stain is to prevent the colour of the methyl green from coming out in the alcohol.

If (Heidenhain, *Arch. f. mik. Anat.*, xxxv, 1890, p. 173; *Zeit. f. wiss. Mik.*, vii, 3, 1890, p. 357) the alcohol used for washing out be slightly alkaline, the stain of the fuchsin will become relatively pale in the result, and the stain of the methyl green relatively strong; whilst a slightly acid reaction of the alcohol will produce the opposite effect. The energy of coloration of the fuchsin is often found to become weakened in kept solutions; it may be restored by adding very dilute acetic acid until a marked intensification of the red tint of the mixture is perceived.

The precautions detailed above are not sufficient to ensure the correct working of the stain, particularly as regards the study of "attraction-spheres" and other achromatic elements. M. Heidenhain (*Ueber Kern und Protoplasma*, Engelmann, 1892, p. 116; see also *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 202, a very full report) gives the following instructions :—Preparations made with the usual mixture are liable to fade; by acidifying the mixture a stronger and more sharply selective stain is obtained, which does not fade. But too much acid must not be added, as this would cause a staining of the interfilar substances. Heidenhain's directions are as follows:

Take some of the "commercial stock solution" (by which appears to be meant the undiluted mixture as given above), and dilute it with distilled water in the proportion of about 6 parts of the mixture to 400 of water. Fill two beakers with distilled water, and add to each a few drops of the diluted solution, so as to bring them both up to the same tint. The coloured liquid thus obtained will show, at the same time, a red tint owing to the rubin, a yellow tone owing to the orange, and a greyish tone derived from the methyl green. Now add to one of the beakers, with continual agitation, drop by drop, a 1 : 500 solution of acetic acid until the colour of the liquid turns to a strong crimson, the former yellow tone disappearing, and the grey of the methyl green becoming less marked. The two beakers serve as standards for the degree of acidity that should be given to the staining bath. The dilute solution that you made by diluting the original stock solution * is now acidified with the dilute acetic acid, added drop by drop with continual agitation, and from time to time a few drops are taken from it and added to a beaker of distilled water until there is obtained the crimson tint of the first test-beaker. Addition of acid should then cease, and the staining bath is ready. If the preparations should not turn out quite satisfactory a small further quantity of acid may be added.

Before staining, sections should be treated for a couple of hours with 0·1 per cent. acetic acid, then for ten to fifteen minutes with officinal tincture of iodine, and be rinsed with alcohol before bringing into the stain, in which they should remain for twelve to eighteen hours. The treatment with acid is necessary in order to ensure having the sections *acid* on mounting in balsam. The primary object of the iodine is to remove any sublimate from the preparations (Heidenhain's descriptions refer to sublimate objects, but Ehrlich-Biondi mixture will work with chrom-osmium objects); but it also enhances the power of staining of the chromatin with methyl green, and produces a more selective staining of protoplasmic elements.

---

* "Das aus der Stammlösung durch Verdünnung gewonnene Quantum."

Nor will these precautions always suffice to ensure correct results. In *Arch. f. mik. Anat.*, xliii, 3, 1894, p. 430, M. HEIDENHAIN adds that it is so highly important to attain and maintain exactly the right degree of acidity, that further precautions are necessary. The solution must not be filtered, for filtration may render it less acid. If it has been kept for some time a little more acid must be added; for it will have dissolved traces of glass, which is an alkaline body. HEIDENHAIN therefore recommends that it be preserved in rubber bottles.

The following instructions for acidifying the EHRLICH-BIONDI mixture, also due to M. HEIDENHAIN, are from a paper of WARBURG's (quoted from *Zeit. f. wiss. Mik.*, xi, 3, 1894, p. 383). To 2 c.c. of the Biondi mixture (1 : 30) (by this is presumably meant the original mixture as given above, but diluted with only 30 volumes of water instead of 60) add 40 c.c. of distilled water, 3 c.c. of 0·5 per cent. solution of Säurefuchsin, and 0·2 c.c. of one-fifth per cent. solution of acetic acid (or, according to GROUVEN, *op. cit.*, xii, 3, 1896, p. 379, four drops). [Grouven here speaks of the mixture as "Triacid" (see next section): there is a deplorable confusion in the nomenclature of these stains.]

The characters of the stain when successful are much as follows:—The nuclear staining is very sharp and good, the chromatic elements being coloured of a somewhat slaty blue. Cytoplasm is of a more or less violet or more or less orange red, and caryoplasm is of the same colour as the cytoplasm, but of a lighter tone. Cell membranes, nuclear membranes, achromatic fibrillar structures, the Nebenkern when present—in a word, all the denser protoplasmic structures, are stained of the same colour as the hyaloplasm, but darker. After a very careful study of this admired stain, I find I cannot recommend it for any but the most special objects. It must in the first place, I think, be acknowledged that a reagent that can neither be prepared nor preserved without the minute precautions detailed above, is something much too great and good for human nature's daily food. The stain is a very fine one when successful, but the most minute precautions will not ensure its being successful. It is very capricious, it seldom gives the same result twice running. The correct result should be a precise chromatin stain, combined with a precise stain of the plastin element of cytoplasm by the Säurefuchsin. Now the least defect or excess of acidity causes the plasma stain of the Säurefuchsin to become a diffuse one, instead of being sharply limited to the plastin element. And the methyl green, being very little resistent to alcohol, goes out of the chromatin with such rapidity during the dehydration, that there is always danger of the chromatin stain being lost altogether. For this reason the stain will only work with very thin sections: to be quite sure of good results, the sections should be of not more than 3 μ in thickness, and if they are over five the desired results are almost hopeless. The preparations keep very badly; the majority of mine at any rate have become spoilt sooner or later, sometimes after only a few days. I admit that the method has its *raison d'être* for the very special objects for which it was imagined,—for the researches on cell-granulations for which EHRLICH employed the three colours, or for the researches on the plastin reticulum of cytoplasm, for which MARTIN HEIDENHAIN employed the mixture; and for similar objects. But to recommend it and to use it, as has been done by many workers, as a general stain for sections, applicable to ordinary work, is nothing but mischievous exaggeration. Far from having the qualities that should be possessed by a normal section-stain, the Ehrlich-Biondi mixture is highly unfitted for ordinary work.

307. EHRLICH'S **Triacid Mixture.**—According to a custom which, I believe, originated with Ehrlich himself, and which would, perhaps, be better honoured in the breach than the observance, the name of Triacid ("Triacidlösung") has been given to a mixture of the same three dyes as in the EHRLICH-BIONDI mixture, but in such proportions that the "acid" colours therein have a larger share assigned to them. The denomination is improper, for the mixture contains only two "acid" colours, methyl green being a strongly "basic" colour. The following is a recent formula of EHRLICH, communicated to REINBACH (quoted from *Zeit. f. wiss. Mik.*, xi, 3, 1894, p. 259).

Orange G, sol. sat. aq.	120
Säurefuchsin ,,	80
Methyl green ,,	100
Distilled water	300
Absolute alcohol	180
Glycerin	50

Care must be taken that the solutions be absolutely saturated before mixing. The mixture must never be shaken, the quantity necessary for use at any time must be carefully taken off from the top of the stock by means of a pipette. With these precautions the mixture will keep for years.

I have not made up this mixture, but have examined a triacid solution procured from Grübler and Co. Its qualities and defects appear to be very much those of the Ehrlich-Biondi mixture. It appears to be a more powerful, but perhaps less delicate stain, and the methyl green appears to have more resistance to alcohol, so that it may perhaps give better results for ordinary work. I have obtained some powerful stains of spindles with it.

As noted in the last section, the modified form of the Ehrlich-Biondi mixture there given has been spoken of as "Triacid."

308. Rawitz' **Adjective Plasma Stains.**—It has been discovered by Rawitz that by means of appropriate mordants certain basic anilins, which by the usual methods of regressive staining are pure chromatin stains, may be made to afford a pure plasma stain—one not affecting chromatin at all. He has succeeded with safranin, fuchsin, methyl violet, gentian violet, and emerald green. The process is as follows:—Sections of material fixed in liquid of Flemming or other chromic mixture (I find that liquid of Hermann will also give the reaction) are put for twenty-four hours into a 20 per cent. solution of tannin in water (this solution must be made in the cold). They are then washed and put for two or three hours into a solution of tartar emetic of from 1 per cent. to 2·5 per cent. strength, kept at a temperature of 37° C., or for twenty-four hours into the same solution at the normal temperature. They are then carefully washed, and are stained for twenty-four hours in one of the above-named dyes, and either differentiated in the usual way with alcohol, or by treating them for from two to twenty-four hours with a 2·5 per cent solution of tannin; they are then cleared, and mounted in the usual way.

Successful preparations show an "inversion" of the stain; the nuclear chromatin remains perfectly colourless, while cytoplasm and intercellular substances are stained. According to Rawitz, spindles are well stained, and "centrosomes" and "attraction spheres" are stained with a sharpness that he has not been able to attain to by any other method.

The instructions given by RAWITZ (*Sitzb. Ges. naturf. Freunde, Berlin*, 1894, p. 174; *Zeit. f. wiss. Mik.*, xi, 4, 1895, p. 503; and his *Leitfaden f. hist. Untersuchungen*, Jena, 1895, p. 76) are not sufficient to ensure success. Rawitz at the time of publishing these instructions had only tried the process on the testis of *Salamandra*. I find that the times necessary vary very much with different objects, and that the results also vary very greatly with different objects. I have only succeeded in obtaining a pure "inversion" by much abridging the baths, especially the staining bath. And I have only succeeded in obtaining a pure "inversion" of the stain with testicular material. As to the value of the process, which RAWITZ commends warmly, I am inclined to regard it as a technical curiosity. I have found it to give, in some instances, an extraordinarily intense stain of polar corpuscles, and think it may possibly be of some use in this respect with testicular material. With sections of tissues in general I do not think it will give tolerable results at all; for it stains intercellular substances with such intensity that it is frequently difficult to distinguish anything at all in the sections. It also seems to me that the tissues suffer under the treatment to a remarkable degree. It is one of the dirtiest stains I have ever seen; every slightest speck of dirt that may be present in the preparations is singled out by the dye, and coloured with remarkable intensity. Many of my preparations have not given the "inversion," but show the chromatin stained in the usual way, and a plasma stain besides.

Theoretically this curious "inversion" of the stain is very interesting. I can at present suggest no further explanation of it than that put forth in § 214.

309. Congo Red (Congoroth) see Griesbach, in *Zeit. f. wiss. Mik.*, iii, 3, 1886, p. 379).—Also an "acid" colour, in the sense in which that appellative is given to Säurefuchsin, &c. The aqueous solution, however, has a neutral or alkaline reaction. It becomes blue in presence of the least trace of free acid (hence Congo is a valuable reagent for demonstrating the presence of free acid in tissues; see the papers quoted, l. c.). A stain much of the same nature as Säurefuchsin, and like it seems to be at present chiefly useful in staining axis-cylinders. See the chapter on Nerve-Tissue, in Part II. It may also be used for staining some objects during life. See *ante*, § 213. I have tried it as a general plasma stain, and cannot recommend it, as the stain does not keep. Note that this colour is not to be confounded with other Congos, as Congo yellow, or Brilliant Congo.

310. Benzopurpurin.—According to Griesbach (l. c.), another "acid" colour very similar in its results to Congo red. It has been warmly commended as a plasma stain, to follow a hæmatein stain or the like. I am bound to say that after some trouble I have been unable to obtain any results whatever with it. See, however, ZSCHOKKE (ibid., v, 4, 1888, p. 466), who recommends **Benzopurpurin B**, and says that weak aqueous solutions should be used for staining, which is effected in a few minutes, and alcohol for washing out. **Deltapurpurin**, a more purple red, has, it is said, similar properties, and may be used in the same way. The solution (aqueous) should be moderately concentrated, and allowed to act for a minute or two.

311. Neutral Red (Neutralroth) (EHRLICH, *Allg. med. Zeit.*, 1894, pp. 2, 20; *Zeit. f. wiss. Mik.*, xi, 2, 1894, p. 250; GALEOTTI, ibid., p. 193). A recently discovered colour, which may be procured from Grübler and Co. Up to the present it has only been employed for *intra-vitam* staining. Tadpoles kept for a day or two in a solution of 1 : 10,000 or 100,000 absorb so considerable a quantity of the colour that all their tissues appear of a dark red. The stain is limited to cytoplasmic granules (EHRLICH), and to the contents of mucus-cells (GALEOTTI).

312. Biebricher Scharlach (BIEBRICH SCARLET), a diffuse bright red stain, may be useful as a contrast stain. See Griesbach, *Arch. f. mik. Anat.*, xxii, p. 132).

313. The Eosins, found in commerce under the names of **Eosin, Saffrosin, Primerose Soluble, Phloxin, Bengal Rose, Erythrosin, Pyrosin B, Rose B à l'Eau**, etc., are all phthalëin colours. The preparations indicated by these names are not quite identical in their properties, but vary according to the different modes of manufacture. Most of them are soluble both in alcohol and in water, but some only in alcohol (*"Primerose à l'Alcool"*).

They are all diffuse stains, formerly much used as contrast stains, less so now.

Their chief use is in combinations or mixtures, to be given further on. For **Bengal Rose** see GRIESBACH, *Zool. Anz.*, No. 135, 1883, p. 172.

Eosin is a specific stain for red blood-corpuscles, and hence is much used in the study of blood, for which see in Part II.

It is also specific for certain granules of leucocytes, for which also see Part II.

The yolk of some ova takes the stain strongly, so that it is useful in some embryological researches.

I at one time used eosin as a contrast stain to gentian violet, but have given up that combination, as I now get better results in other ways.

314. Methyl Green and Eosin (CALBERLA, *Morph. Jahrb.*, iii, 1877, Heft 3, p. 625).—Mix 1 part of eosin with 60 parts of methyl green, and dissolve the mixture in warm 30 per cent. alcohol.

Sections stain in this solution in five or ten minutes; they should be quickly washed in successive alcohols, and mounted in balsam or glycerin.

Methyl Green and Eosin (LIST, *Zeit. f. wiss. Mik.*, ii, 1885, p. 147).—Stain for a few minutes in a mixture of three parts of absolute alcohol with one part of aqueous solution of eosin (0·5 per cent.), wash, and stain for five minutes in 0·5 per cent. aqueous solution of methyl green. Wash, dehydrate, clear, and mount in balsam. The preparations do not keep well in glycerin.

The method may be varied (l. c., p. 150) by diluting the methyl green solution with fifty volumes of water, and staining for twenty-four hours.

It may also be varied by diluting the original methyl green solution with three volumes of absolute alcohol.

The preparations should not be left in the alcohol used for dehydrating after the colour of the eosin has begun to reappear.

315. Methylen Blue and Eosin (Chenzinsky, quoted from *Zeit. f. wiss. Mik.*, xi, 2, 1894, p. 260).

Methylen blue, sol. sat. in water	40
Eosin, 0·5 per cent. in 70 per cent. alcohol	20
Distilled water, or glycerin	40

This solution will only keep for about eight days.

It has been recommended by good workers as a specific stain for blood. I find it will work with sections, but do not recommend it for that purpose. The eosin is diffuse in the cytoplasm.

The mixture of Pianese (*Zeit. f. wiss. Mik.*, xi, 3, 1894, p. 345) contains the same ingredients in the same proportions, with the addition of a considerable proportion of carbonate of lithia.

See also the mixture of Bremer, *Arch. f. mik. Anat.*, xlv, 1895, p. 433; or *Zeit. f. wiss. Mik.*, xii, 3, 1896, p. 380.

316. Light Green (Lichtgrün S. F.). An acid colour, soluble in water or alcohol, and a valuable plasma stain.

Benda (*Verh. physiol. Ges. zu Berlin*, Dec. 18th, 1891, Nos. 4 u. 5; see also *Zeit. f. wiss. Mik.*, viii, 4, 1892, p. 516) proceeds as follows (testes of Mammalia):—Sections stained for twenty-four hours in anilin-water safranin solution, then for about half a minute in a solution of 0·5 grm. "Lichtgrün F. S." or Säureviolett (Grübler) in 200 c.c. of alcohol, dehydrated, and mounted in balsam. Chromatin red, archiplasma bright green (or violet), centrosomata and "Spitzenknopf" (of spermatozoa) sometimes green, sometimes red. The chromatoid "Nebenkörper" of Hermann stains red.

I have carried out this process both with the Lichtgrün and the Säureviolett. It gives one of the most beautiful stains known to me. The Säureviolett gives perhaps the more brilliant preparations and seems to be rather easier to carry out, as it may be allowed to act rather longer than the Lichtgrün. The process in either form is a rather delicate one to carry out, and requires very thin sections. The Lichtgrün stain unfortunately does not keep very well; I find my preparations much faded after two years, but they will keep sufficiently for many months at all events.

317. Malachite Green (syn. **Solid Green, Victoria Green, New Green, Benzoyl Green, Fast Green**). A basic colour, which has been used as a plasma stain for the ova of *Ascaris* by **van Beneden** and **Neyt** (see in Part II, "Cytological Methods") These authors used it for glycerin preparations. I have tried it for balsam sections, and find that it cannot be used, as the stain is not sufficiently resistent to alcohol.

318. Iodine Green (HOFFMANN'S **Grün**"), see GRIESBACH, *Zool. Anz.*, No. 117, vol. v, 1882, p. 406.—The high praise accorded to this stain by Griesbach has not been justified by the experience of other workers. The colour is now no longer manufactured for industrial purposes, but may be obtained of excellent quality from C. A. F. Kuhlbaum's Chemische Fabrik, Berlin, S.O. (*Zool. Anz.*, No. 130, 1883, p. 56).

319. Thiophen Green (Thiophengrün), see KRAUSE, *Intern. Monatsschr. f. Anat.*, &c., iv, 1887, Heft 2.

320. Anilin Green is said to have a special affinity for mucous gland cells, and other qualities; but I have not been able to identify the colour mentioned by authors. Recent chemical treatises make no mention of any dye bearing this name, which is, therefore, presumably an erroneous attribution.

321. Quinoleïn Blue (Cyanin, Chinolinblau; v. Ranvier, *Traité*, p. 102).—Quinoleïn is said to have the property of staining fatty matters an intense blue.

It is useful for staining Infusoria, which in dilute solution it stains during life. On this point see the methods of Certes (*post*, Part II).

322. Indulin and **Nigrosin.**—The **Indulins** are a group of dyes related to the base violanilin. They may be either "basic" colours, or "acid" colours, according as they have or have not by suphonation been converted into suphonic acids. The soluble indulins of commerce are generally mono- and disulphonic acids (BENEDIKT and KNECHT, *Chemistry of Coal-tar Colours*, p. 187). They occur under the brands (not strictly synonyms) of **Indulin, Nigrosin, Indigen, Coupier's Blue, Fast Blue R, Fast Blue B, Blackley Blue, Guernsey Blue, Indigo substitute**. According to BEHRENS the name Indulin is generally given to a bluish brand, and that of Nigrosin to a blacker one.

Nigrosin, used with sublimate material, I find stains both nuclei and cytoplasm, the chromatin strongly (see § 281). I do not consider it a very good plasma stain. It will not give the stain at all with chromo-osmium material.

According to CALBERLA (*Morph. Jahrb.*, iii, 1877, p. 627), *Indulin* dissolves into a dark blue solution in warm water or in dilute alcohol. For staining, the concentrated aqueous solution should be diluted with six volumes of water. Sections will stain in the dilute solution in five to twenty minutes; they may be washed in water or in alcohol, and examined either in glycerin or oil of cloves.

The peculiarity of this stain is said by Calberla to be that it never stains nuclei; the remaining cell-contents and intercellular substance are stained blue. In its general effects it resembles quinoleïn blue, and is exactly the opposite of methyl green. The stroma of tendinous tissue, for instance, stains of a fine blue, the connective tissue that surrounds the bundle hardly at all, and the tendon-corpuscles of Ranvier remaining perfectly colourless, stand out as white stellate figures on a blue ground. As far as I have been able to control Calberla's assertions, they appear to me correct. Used on Flemming material, Indulin seems to me a pure but weak plasma stain, leaving nuclei entirely unaffected, and possessing therefore opposite qualities to those of Nigrosin (see § 281). For Flemming material it will evidently be well to employ as strong a solution as possible. I see no special good in it.

323. EHRLICH'S **Indulin-Aurantia-Eosin**, or **Acidophilous Mixture**, or **Mixture C**, or **Mixture for Eosinophilous Cells** (from the formula kindly sent me by Dr. Grübler).—Indulin, aurantia, and eosin, of each two parts; glycerin, thirty parts. This gives a very thick, syrupy solution. To use it, cover-glass preparations may be floated on to it; or sections on slides may have a few drops poured on to them, the slide being laid flat till the stain has taken effect (twenty-four hours for Flemming material). This mixture was imagined for the purpose of obtaining a specific stain of certain granules of leucocytes. It has been pointed out by NIKIFOROW (*Zeit. f. wiss. Mik.*, viii, 2, 1891, p. 189; and xi, 2, 1894, p. 246) that it is also available for staining sections. I find this is the case. With Flemming material it gives a powerful and good stain, which is much more resistent to alcohol than that of the Ehrlich-Biondi mixture, and is therefore much more adapted to ordinary work. The stain with my objects is a blue one, in general effect like a hæmatein stain, the eosin and aurantia only coming into play with certain elements. Chromatin in my preparations is of a very dark blue, cytoplasm being of a lighter blue (except where it has taken the stain of the aurantia or eosin). It will thus be seen that the Indulin in this combination behaves in a manner quite opposed to its behaviour when used alone (see last section). The stain is said to keep well.

324. Safranin and Indigo-Carmine (KOSSINSKI, *Zeit. f. wiss. Mik.*, vi, 1, 1880, p. 61).—Stain sections ten to twenty minutes in saturated aqueous solution of indigo-carmine, wash with water and with alcohol, and stain with safranin (0·5 per cent. in dilute alcohol), dehydrate and mount.

Safranin and Nigrosin is a combination also recommended by the same author. Stain for three to five minutes in 0·1 per cent. aqueous solution of nigrosin, and proceed as before.

N.B.—These times are stated for sublimate or alcohol material. I find they do not apply at all to chrom-osmium material. With this neither nigrosin nor indigo-carmine will give a stain.

325. Anilin Blue.—Under this title are comprised various derivatives of the base rosanilin. They occur under the names **Spirit Soluble Blue (Bleu Alcool), Gentian Blue 6 B, Spirit-Blue 0, Opal Blue, Bleu de Nuit, Bleu Lumière, Parma Blue, Bleu de Lyon.** Receipts of the older authors for staining with "Anilin blue" should I think, be disregarded, as it would probably now be impossible to obtain the colours used by them or even to ascertain what colour was meant by them.

The only one of the above-mentioned colours of which I have any personal knowledge, or that appears really valuable, is Bleu de Lyon. (Some authors give the names Bleu de Nuit and Grünstichblau as synonyms of Bleu de Lyon.) I find that though there is nothing very specific in its action, it is a very good plasma stain. It is a fairly true plasma stain, for though in a strong stain it will stain chromatin, yet in a light stain it will stain cytoplasm first, and thus works very well with carmine or safranin as a nuclear stain, leaving the chromatin of a perfectly pure red. I find it gives very good differentiations of nerve tissue, and of cartilage (as has already been pointed out by BAUMGARTEN and by JACOBY). The older mode of using it (MAURICE and SCHULGIN) was to stain in bulk with it after borax-carmine, using a very dilute alcoholic solution. Baumgarten and Jacoby stain sections in a 0·2 per cent. alcoholic solution. I have experimented with safranin as the chromatin stain, and obtained some fair results. But I do not think the combination can be recommended. For if you stain first with the blue, the safranin will extract it if allowed to act for more than a short time. And if you stain first with the safranin, the blue will extract it very quickly. The stain is a somewhat opaque one, and the results when obtained appear to me to be not comparable for delicacy with those given by Wasserblau (next §).

326. Methyl Blue.—Under this title are comprised some other derivatives of the base rosanilin. They are acid colours, being mostly salts of triphenylrosanilin- and triphenylpararosanilin-trisulphonic acid. Here belong the dyes sold as **Methyl Blue, Cotton Blue, Water Blue** (**Wasserblau**), **Methyl Water-Blue, China Blue** (**Chinablau**), **Soluble Blue.**

Amongst these **Water Blue** (**Wasserblau**), appears to possess some useful properties. According to MITROPHANOW (quoted from *Zeit. f. wiss. Mik.* v, 4, 1888, p. 513), used in concentrated aqueous solution it gives a very good double stain with safranin. It is very resistent to alcohol. Using the Wasserblau first, and then the safranin, I have had some interesting results, and as the process is easy to carry out I think it may be recommended. The Wasserblau must be used first, as if used after the safranin it will destroy the stain in a short time. With chrom-osmium material, twelve to twenty-four hours in the blue, and four or five in the safranin, may not be too much.

MANN (*op. cit.*, xi, 4, 1894, p. 490) has used it in conjunction with eosin for staining ganglion cells. For the somewhat complicated details of the process, see the place quoted.

327. Anilin Blue-black.—A preparation cited under this name has been recommended by BEVAN LEWIS and others for nervous tissue. Unfortunately these authors have not given the chemical description of the colour used by them, so that it is not possible to ascertain whether they mean the **Blue-black B** of the oxyazo series, or the **Anilin black** of Lightfoot, also known under the names of **Nigranilin** and **Noir Colin.**

328. Carmine Blue (**Bleu Carmin Aqueux,** prepared by Meister, Lucius and Brunig, at Höchst-a-M.). JANSENS (*La Cellule*, ix, 1, 1893, p. 9) has shown that this colour possesses a special affinity for the parts of cytoplasm that are undergoing cuticular differentiation. He uses it in alcoholic solution acidified. The stain will bear mounting in balsam.

329. Violet B (S. MAYER, *Sitzb. d. k. k. Akad. d. wiss. Wien*, iii Abth., February, 1882).—This colour is a methyl violet prepared by Bindschedler and Busch of Bâle, and by the Aktienfabrik für Anilinfarben at Berlin. Used in solutions of 1 grm. of the colour to 300 grms. of 0·5 per cent. salt solution, and with *fresh tissues* that have not been treated with any reagent whatever, this colour gives a stain so selective of the elements of the vascular system that favorable objects, such as serous membranes, appear as if injected. The preparations do not keep well; acetate of potash is the least unsatisfactory medium for mounting them in.

330. Säureviolett, see § 316.

331. Benzoazurin may be made to give either a diffuse or a nuclear stain, according to Martin (see *Journ. Roy. Mic. Soc.*, 1890, p. 114).

332. Baumgarten's Fuchsin and Methylen Blue (*Zeit. f. wiss. Mik.*, i, 1884, p. 415).—Stain sections (of chromic objects) for twenty-four hours in a stain made by adding 8 to 10 drops of concentrated alcoholic solution of fuchsin to a watch-glassful of water. Rinse with alcohol, and stain for four or five minutes in concentrated aqueous solution of methylen blue, wash out with alcohol for five to ten minutes, and clear with clove oil. Nuclei red, tissues blue, the fuchsin having been driven out of the tissues by the methylen blue, a result which is *not* attained by washing with alcohol alone, either pure or acidified.

333. Artificial Alizarin (Rawitz, *Anat. Anz.*, xi, 10, 1895, p. 294). Rawitz has worked out a process of obtaining a double stain (chromatin and cytoplasm being stained of different colours) by means of artificial Alizarin, or Alizarin-cyanin. The process is an adjective one, requiring the use of special mordants only to be obtained from the colour manufacturers, and is extremely complicated and very lengthy, taking at least several days. As the results, to judge from the description of Rawitz, do not seem to possess a single point of superiority over those that may be obtained in half an hour by use of two or more colours in succession, or in combination according to known methods, the process would seem to be superfluous.

CHAPTER XIX.

OTHER ORGANIC STAINS AND COMBINATIONS.

334. Kernschwarz (Platner, *Zeit. f. wiss. Mik.*, iv, 3, 1887, p. 350; *Journ. Roy. Mic. Soc.*, 1888, p. 675).—Kernschwarz is a black liquid of unknown composition, prepared in Russia. It may be obtained from Grübler (address § 216). Platner's method of using it was as follows:—Sections (sections only) may be stained in a tolerably strong dilution of the concentrated liquid, and washed out (it may be for some hours) in an alkaline aqueous liquid. Dilute ammonia will do, but it is better to take a not quite saturated solution of carbonate of lithium (you may take a saturated solution, and dilute it with three, four, or more volumes of water). The result is a nuclear stain in the cytological sense; nuclear figures of division are stained deeply, resting chromatin less deeply or not at all, cytoplasm unstained or faintly grey. A peculiarity of this stain, on which much stress was laid by Platner in his first announcement of the colour, is that it stains also the *Nebenkern* (which safranin and the other basic anilins do not do when employed in the usual way).

Platner, on the whole, recommended this colour as a *chromatin* stain. As stated in former editions, I do not find that it possesses any very brilliant qualities as a chromatin stain. But I consider that employed *as a plasma stain* it has very important qualities. It is one of the most useful plasma stains that I am acquainted with. I do not say one of the most beautiful, for the sober neutral-tint stain it gives is not æsthetically seductive. I use it as follows:

Sections (of chrom-osmium material—sublimate material does not give good results) are fixed on slides and treated with Kernschwarz until they become of a somewhat yellowish grey. If the material is fresh, the required depth of stain may be obtained in a few minutes, and in that case it may be well to first dilute the Kernschwarz with about ten volumes of water, so as not to run the risk of overstaining. If the material is not fresh, that is if it has been kept for some months, it will be necessary to stain for twenty-four hours in the undiluted liquid. The differentiation in an alkaline fluid prescribed by Platner is omitted. After staining, I rinse in water, and stain for twenty-four hours or more in a chromatin stain, safranin by preference, but gentian violet, Victoria blue, or a hæmatein stain will do. I extract the safranin with alcohol, acidulated or neutral according to circumstances, followed by clove oil, and mount in the usual way. This process gives a nearly pure double stain. In nuclei the chromatin and nucleoli are of a dark red (which appears rather brownish because the Kernschwarz has stained the plastin sheath of these elements), whilst the plastin element of cytoplasm is of a rich purple grey.

The enchylema or hyaloplasm of nuclei and cytoplasm is colourless, or only lightly stained; spindles are faintly stained; spindle-relics, both equatorial and polar ("Nebenkerne," or "Spheres" of some authors) are deeply and accurately stained. As I have explained elsewhere (*La Cellule*, xi, 1, 1895, p. 31, and xi, 2, 1896, p. 257) this method is the best I have been able to discover for the study of these elements. But I have also found it to be excellent for the study of very various tissues, and as it is perfectly safe and very easy to execute, I think it may be recommended as a method of very general applicability.

The stain of Kernschwarz is stated to be a good one for preparations that it is desired to photograph.

335. Orchella (Orseille) (WEDL, *Arch. f. path. Anat.*, lxxiv, p. 143; *Journ. Roy. Mic. Soc.*, ii, 1879): for an account of this substance *vide* COOLEY'S *Cyclopædia*, *sub voce* "Archil;" and see FOL, *Lehrb.*, p. 192, and former editions of this work.

336. Orcein (ISRAEL, *Virchow's Archiv*, cv, 1886, p. 169; *Journ. Roy. Mic. Soc.*, 1887, p. 514, and ISRAEL, *Prakticum der path. Hist.*, 2 Aufl., Berlin, 1893, p. 72).—Orcein (not "orcin") is a vegetable dye obtained from the tinctorial lichen, *Lecanora parella*, and is not to be confused with *orcin*, another derivative of the same lichen. It is said to unite in itself the staining properties of the basic and acid stains, and also the combination of two contrast colours. Israel stains sections in a solution containing 2 grms. of orcein, 2 grms. of glacial acetic acid, and 100 c.c. of distilled water, washes in distilled water, and passes rapidly through absolute alcohol to thick cedar oil, in which the preparations remain definitively mounted. Nuclei blue, protoplasm red.

For the specific staining of elastic and connective tissue by means of this reagent, a subject which has been carefully worked out by Tänzer and Unna, see the paragraphs on the Connective Tissues in Part II.

As far as I can see, this dye is not likely to be of any use in ordinary work. I have not obtained a good solution in the acetic acid. Using a 1 per cent. solution in absolute alcohol, I have obtained a faint plasma stain, the most conspicuously stained element of the cells being the Nebenkern, which is well brought out. The colour may perhaps be useful for the study of this element.

337. Purpurin, see Ranvier's *Traité technique*, p. 280; Duval's *Précis de Technique histologique*, p. 221; and Grenacher's formula in *Arch. f. mik. Anat.*, xvi, 1879, p. 470.

338. Indigo.—Indigo is employed in histology in the form of solutions of so-called indigo-carmine, or sulpindigotate of soda or potash. The simple aqueous solution gives a diffuse stain, and is therefore not capable of being usefully employed *alone*. It is, however, of use when employed to bring about a *double* stain in conjunction with carmine. Though it has no selective preference for nuclei or protoplasm, it possesses to a high degree the property of imparting different hues and intensity of stain to different tissues; and the nuclei being brought out by carmine, preparations are obtained of a diagrammatic clearness that is not afforded by carmine alone. Some processes for double-staining with carmine are mentioned below. I have tried to use indigo-carmine in combination with safranin, and have not succeeded, but see § 324.

Indigo-carmine is found in commerce. The reader who may desire to prepare it himself will find the necessary directions in *Arch. f. mik. Anat.*, x, 1874, p. 32, and in *Journ. Roy. Micr. Soc.*, ii, 1879, p. 614.

Thiersch's Oxalic Acid Indigo-carmine (see *Arch. f. mik. Anat.*, i, 1865, p. 150).

Carmine Combinations.

339. Seiler's Carmine followed by Indigo-Carmine (*Am. Quart. Mic. Journ.*, i, 1879, p. 220; *Journ. Roy. Mic. Soc.*, ii, 1879, p. 613).—Stain in borax-carmine, wash out with HCl alcohol, wash out the acid, and after-stain in an *extremely dilute* alcoholic solution of indigo-carmine (two drops of saturated aqueous solution added to an ounce of alcohol and filtered).

I find this method gives good results when applied to sections, but very bad results if it be attempted to stain in the mass with the indigo. The indigo over-stains the superficial layers before it has penetrated to the deeper layers. The instructions given refer to sublimate material or the like; I find chrom-osmium material will not take the stain at all.

340. Merkel's Carmine and Indigo-Carmine in One Stain (MERKEL, *Unters. a. d. anat. Anst. Rostock*, 1874; *Month. Mic. Journ.*, 1877, pp. 242 and 317).

Authors (MERKEL, *loc. cit.;* NORRIS and SHAKESPEARE, *Amer. Journ. Med. Sc.*, January, 1877; MERKEL, *Mon. Mic. Journ.*, 1877, p. 242; MARSH, *Section Cutting*, p. 85; BAYERL, *Arch. f. mik. Anat.*, xxiii, 1885, pp. 36, 37; MACALLUM, *Trans. Canad. Instit.*, ii, 1892, p. 222; *Journ. Roy. Mic. Soc.*, 5, 1892, p. 698) are unanimous in stating that successful preparations show a most richly differentiated and yet very precise colouring. According to Bayerl, the stain is quite specifically elective for red blood-corpuscles, which are stained of an apple-green. The ground substance of cartilage and bone stains blue, their cells red.

But the results are very uncertain. Merkel's formula, as has been pointed out by PAUL MAYER (*Mitth. Zool. Stat. Neapel*, xii, 2, 1896, p. 320) is not only highly irrational, and inconvenient to employ, but gives an alkaline fluid that may be injurious to tissues. I agree with him that it should be suppressed.

341. MAYER'S **Carmine (or Hæmatein) and Indigo-Carmine in one stain.**—In the place quoted in the last section, MAYER states that he obtains very good results by taking a solution of 0·1 gramme of indigo-carmine in 50 c.c. of distilled water, or 5 per cent. alum solution, and combining it with from four to twenty volumes of carmalum or hæmalum.

342. Carmine and Anilin Blue (or Bleu Lumière, or Bleu de Lyon).—DUVAL (*Précis de technique microscopique*, 1878, p. 225) proceeds as follows:—Stain with carmine "in the ordinary way;" dehydrate, and stain for a few minutes (ten minutes for a section of nerve-centres) in an alcoholic solution of anilin blue (ten drops of saturated solution of anilin blue soluble in alcohol to ten grammes of absolute alcohol, for sections of nerve-centres). Clear with turpentine, without further treatment with alcohol, and mount in balsam.

The sections should appear of a fine dark violet when taken from the anilin; they are extremely transparent under the microscope—nerve-cells and axis-cylinders reddish violet, blood-vessels bluish violet, and so sharply marked out that the preparations have the aspect of injections. The connective elements are stained of a nearly pure blue, so that it is easy to distinguish them from the nervous elements.

Applicable to all kinds of tissues, but especially to sections of nerve-centres.

Recent authors recommend, instead of anilin blue, bleu de Lyon, dissolved in 70 per cent. alcohol acidulated with acetic acid (MAURICE and SCHULGIN), or bleu lumière, which has hardly any effect on nuclei.

The solutions of both these colours should be extremely dilute for sublimate material, but strong for chrom-osmium material. It is possible to use them for staining in bulk.

BAUMGARTEN (*Arch. f. mik. Anat.*, xl, 1892, p. 512) stains sections (of material previously stained in borax-carmine) for twelve hours in a 0·2 per cent. solution of bleu de Lyon in absolute alcohol, and washes out for about half that time before mounting in balsam. He recommends the process for cartilage and nerve-centres.

343. **Carmine and Malachite Green.**—MAAS (*Zeit. f. wiss. Zool.*, x, 4, 1890, p. 527 ; *Zeit. f. wiss. Mik.*, viii, 2, 1891, p. 205) recommends borax-carmine followed by weak alcoholic solution of malachite green, with a final washing out with stronger alcohol, see also § 317.

344. **Carmine and Picro-nigrosin** (PIANESE) (see *Journ. Roy. Mic. Soc.*, 1892, p. 292).

345. **Carmine and Picric Acid.** See § 299.

Hæmatein or Hæmatoxylin Combinations.

346. **Hæmatoxylin and Picric Acid.**—This excellent combination has been treated of above (§ 299).

347. **Hæmatoxylin and Eosin.**—This is a well-known and popular combination, though some workers prefer the combination of hæmatoxylin with **Benzopurpurin** (§ 310). Objects may be stained with hæmatoxylin (either in the mass or as sections) and the sections stained for a few minutes in eosin. I think it is better to take the eosin weak, though it has been recommended (STÖHR, see *Zeit. f. wiss. Mik.*, i, 1884, p. 583) to take it saturated. Either aqueous or alcoholic solutions of eosin may be used.

HICKSON (*Quart. Journ. Mic. Sci.*, 1893, p. 129) gives the following instructions for staining sections on the slide :—

One hour in a strong solution of eosin in 90 per cent. alcohol, wash with alcohol, and stain for twenty minutes in a weak solution of hæmatoxylin.

This method is most particularly recommendable for embryological sections, as vitellus takes the eosin stain energetically, and so stands out boldly from the other germinal layers in which the blue of the hæmatoxylin dominates.

LIST (*Zeit. f. wiss. Mik.*, ii, 1885, p. 148) stains for twenty-four hours in a solution of three or four drops of Renaut's hæmatoxylic glycerin (§ 259) in 250 c.c. of water, and then for a few minutes in a mixture of one part of 0·5 per cent. aqueous solution of eosin with three parts of absolute alcohol.

See also BUSCH (*Verh. Berl. Phys. Ges.*, 1887); GIERKE (*Zeit. f. wiss. Mik.*, i, 1884, p. 505).

It should be noted that sections should be very well washed before being passed from eosin into hæmatoxylin or the reverse, as eosin very easily precipitates hæmatoxylin.

348. RENAUT'S **Hæmatoxylic Eosin** (FOL'S *Lehrbuch*, p. 196).—Renaut has given from time to time several formulæ for this stain. This one, communicated to Fol by Renaut, is the latest, and I suppress the others.

Take—

Concentrated aqueous solution of potassic eosin (*éosine à la potasse*)	30 c.c.
Saturated solution of hæmatoxylin in alcohol (ought to have been kept some time and to have precipitated)	40 c.c.
Saturated solution of potash alum in glycerin (of a density of about 1·26) . . .	130 c.c.

Mix, and let the mixture stand five or six weeks in a vessel covered with a sheet of paper pierced with holes until the alcohol is evaporated, then filter.

For staining, the solution may be used as it is or diluted. Staining goes on very slowly, and at first the colour is not held by the tissues, but disappears on washing. After some days or weeks, however, it becomes localised and fixed in the tissues. You may then mount in balsam, taking care to employ alcohol charged with a sufficient quantity of eosin. But it is frequently preferable to proceed by mounting the objects in the staining fluid diluted with one to two volumes of glycerin. After a few weeks this mounting medium will have become perfectly colourless through the absorption of the colour by the tissues.

The stain has a specific action on the cells of salivary and gastric glands. Mucus-cells become pale blue; salivary ferment-cells (crescent-cells of Gianuzzi) intense rose.

See also *Comptes Rendus,* 1879, p. 1039 1re sér., and *Arch. de Physiol.*, 1881, p. 640.

Everard, Demoor and Massart (*Ann. Inst. Pasteur,* vii, 1893, p. 166), prepare a similar mixture as follows:—A solution is made with 1 grm. of eosin, 25 grm. of alcohol, 75 of water, and 50 of glycerin. Then 20 grm. of alum are dissolved by the aid of heat in 200 grm. of water, the solution is filtered, and after twenty-four hours there is added to it 1 grm. of hæmatoxylin dissolved in 10 grm. of alcohol. This solution is allowed to stand for eight days, then filtered again, and combined with an equal volume of the eosin solution.

349. Hæmatoxylin and Benzopurpurin (Zschokke).—For this and the combination with **Delta Purpurin,** see *ante,* § 310.

350. Hæmatein and Säurefuchsin.—Get a sharp chromatin stain with hæmalum, then stain for five or ten minutes in 0·5 per cent. aqueous solution of Säurefuchsin, dehydrate and mount. I would only advise this for sublimate material, not for chrom-osmium material.

351. Hæmatoxylin and Säurefuchsin and Orange.—Proceed as above, using for the second stain the following mixture: Säurefuchsin, 1 grm.; orange G, 6 grm.; rectified spirit, 60 c.c.; water, 240 c.c. (from Squire's *Methods and Formulæ,* p. 42). I consider this a very good combination. Ten minutes is rather too much for sublimate sections, and not enough for chrom-osmium sections.

The process described by Cavazzani (*Riforma Med.,* Napoli, 1893, p. 604, *Zeit. f. wiss. Mik.,* xi, 3, 1894, p. 344) is far too complicated to be recommendable.

352. Hæmatoxylin and Safranin.—In this combination, which was used to such good effect by Rabl in his classical researches on nuclei (*Morph.*

Jahrb., x, 1884, p. 215), the safranin is the chromatin stain, and the hæmatoxylin the plasma stain. You stain *very lightly* with hæmatoxylin, so lightly that the stain would not be of any use by itself (Rabl used *very dilute* Delafield's solution, for twenty-four hours); wash out first with water, and then with alcohol acidulated with HCl, then stain for some hours in (Pfitzner's) safranin, and wash out with pure alcohol. Rabl certainly was not far wrong when he wrote, "This method is unequalled by any other." It was certainly one of the best known at that time; but better plasma stains are now known, and for most purposes the method may be considered to be out of date.

Foà (*Festschr. R. Virchow gewidmet*, &c., i, 1891, p. 481; *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 227) prefers using the hæmatoxylin and safranin combined in one solution. You take—

Aq. dest. . . . . ca.	100
Böhmer's hæmatoxylin	25
Safranin, usual 1 per cent. water and alcohol solution	20

Stain sections in a few drops of the mixture for from one to three minutes, wash in water, dehydrate, and mount; or before dehydrating treat with a weak alcoholic solution of picric acid, or, for some cases, of orange. This mixture was specially used for staining marrow in an investigation into the development of blood.

CHAPTER XX.

METALLIC STAINS (IMPREGNATION METHODS).

353. The Characters of Impregnation Stains.—By impregnation is understood a mode of coloration in which a colouring matter is deposited in certain elements of tissues in the form of a more or less finely granular or sometimes even flocculent *precipitate*—the impregnated elements becoming in consequence opaque. By staining, on the other hand, is understood a mode of coloration in which the colouring matter is retained by the tissues in a state of *solution*, showing no visible solid particles under the microscope, the stained elements remaining in consequence transparent. But it should be understood that it is not correct to draw a hard and fast line between the two kinds of coloration. Some of the metallic salts treated of in this chapter give, besides an impregnation, in some cases a true stain. And some of the dyes that have been treated of in preceding chapters give, besides a stain, a true impregnation. Methylen blue, for instance, will give in one and the same preparation an impregnation and a stain; and critical examination of most fairly successful gold chloride preparations will show that the coloration is in places of the nature of a finely divided solid deposit, in others a perfectly transparent stain.

Thus is justified the alternative title that this chapter has always borne—Metallic Stains, *or* Impregnation Methods.

354. Negative and Positive Impregnations.—Impregnations are distinguished as *negative* and *positive*. In a negative impregnation intercellular substances alone are coloured of a deep black or brown or violet, according to the method employed, the cells themselves remaining colourless or very lightly tinted. In a positive impregnation the cells are stained and the intercellular spaces are unstained. (This explanation is the more needful as a directly contrary statement is made in a recent *Lehrbuch*.)

Negative impregnation is *primary* because it is brought about by the direct reduction of a metal in the intercellular spaces. Positive impregnation is *secondary* (in the case of silver nitrate) because it is brought about by the solution in the liquids of the tissues of the metallic deposit formed by a primary or negative impregnation, and the consequent staining of the cells by the new solution of metallic salt thus formed. These secondary impregnations take place when the reduction of the metal in the primary impregnation is not sufficiently energetic (see on these points HIS, *Schweizer Zeit. f. Heilk.*, ii, Heft 1, p. 1; GIERKE, *Zeit. f. wiss. Mik.*, i, p. 393; RANVIER, *Traité*, p. 107).

355. Nature of the Metallic Deposit.—There still exists considerable obscurity as to the nature of the black or brown deposit formed in the intercellular spaces in cases of primary impregnation with a silver salt; v. Recklinghausen held that the silver salt combined with a hypothetical intercellular cement-substance (*Kittsubstanz*), forming a compound that blackens under the influence of light. Other authors refuse to believe in the intercellular cement, and hold either that the coloured lines represent stained cell-membranes, or that the metallic salt combines with the albuminous and saline liquids that surround the cells, and is precipitated in simple intercellular *spaces*. SCHWALBE (*Arch. f. mik. Anat.*, vi, 1870, p. 5), thinks that two cases should be distinguished; the *black* lines that are obtained by the action of very weak solutions for a very short time being due to a true precipitate formed by a reduction of metal in the intercellular liquids, the *brown* lines that are obtained by exposing tissues for a longer period to the action of more concentrated solutions being due to the formation of a compound of metal and cement-substance that becomes brown on exposure to light. (For the history of these questions see GIERKE's *Färberei zu mikroskopischen Zwecken.*) JOSEPH (*Sitzb. d. k. preuss. Akad. d. Wiss. Berlin*, 1888; *Zeit. f. wiss. Mik.*, xi, 1, 1894, p. 42, *et seq.*) has pointed out that the deposit can in no case consist of metallic silver, for it is dissolved by sodium hyposulphite, but it may be an albumino-nitrate or an oxide of silver.

356. Action of Light on Solutions of Metallic Salts.—Stock solutions of metallic salts are generally kept in the dark, or at least in coloured bottles, under the belief that exposure to light spoils them by precipitating the metal in a state of reduction. It has been pointed out in § 33 that in the case of osmic acid not light, but dust is the reducing agent;

and that solutions may be exposed to light with impunity if dust be absolutely denied access to them. I have now good evidence to the effect that the same is the case with other metallic solutions; and the interesting point is raised whether such solutions are not positively improved for impregnation purposes by exposure to light! My able friend Dr. LINDSAY JOHNSON has been examining this question both from a histological and from a photographic point of view, and writes me as follows:

"One may (I find by experiment) state as a rule without exception that all the solutions of the chlorides and nitrates of the metals will keep indefinitely in clean white stoppered bottles in the sunlight; and as far as osmium, uranium, gold and silver, and platinum are concerned, actually improve or ripen by a good sunning. All photographers tell me their papers salt more evenly by old well-sunned silver nitrate than by a fresh solution kept in the dark; and I go so far as to say that this is one of the reasons why gold stains are so unsatisfactory." I have not been able myself to perform any experiments for the purpose of confirming the hypothesis of ripening by sunning, but I must say that it appears to me very plausible and deserving of further inquiry.

357. **State of the Tissues to be impregnated.**—It has been pointed out in earlier chapters that the majority of histological stains are not obtained with fresh tissues, but with tissues that have been more or less charged with metallic salts, or otherwise changed in their composition by the action of fixing and preservative reagents. The contrary is the case with metallic impregnations: perfectly *fresh* tissues—that is, such as are either living or at all events have not been treated by any reagent whatever—are those in general that impregnate with the greatest ease and precision. Most impregnations will not succeed at all with tissues that are not fresh in the sense above explained.

Silver.

358. **Silver Nitrate: Generalities.**—This is the most commonly used salt of silver. The general principles of its employment are so well stated by RANVIER (*Traité*, p. 105) that I cannot do better than abstract his account.

Silver nitrate may be employed either in solution or in the solid state. The latter method is the less frequently employed, but is easy and gives good results. It is useful for the study of the cornea and of fibrous tissue, but is not suitable for epithelia. For the cornea, for instance, proceed as follows:—The eye having been removed, a piece of silver nitrate is quickly rubbed over the anterior surface of the cornea, which is then detached and placed in distilled water; it is then brushed with a camel's-hair brush in order to remove the epithelium. The cornea is then exposed to the action of light. On subsequent examination it will be found that the silver nitrate, which was dissolved by the liquid that bathes the surface of the cornea, has traversed the epithelium and soaked into the fibrous tissue, on the surface of which it is reduced by the action of light. The cells of the tissues will be found unstained.

Silver nitrate is generally employed in solution in the following manner:—A 1 per cent. solution is taken, to which two, three, or four volumes of water are added according to circumstances. The mode of employment varies in its details according to circumstances, a point which is very important to observe. In the case of a membrane such as the epiploön, the membrane must be stretched like a drum-head over a porcelain dish,* and washed with distilled water, in order to remove the albuminates and white blood-corpuscles that are found on its surface; it is then washed with the solution of silver nitrate. In order to obtain a powerful stain it is necessary that this part of the operation be performed in direct sunlight, or at least in a very brilliant light. As soon as the tissue has become white, and has begun to turn of a blackish grey, the membrane is removed, washed in distilled water,

---

* **The Hoggans' Histological Rings** will be found much more convenient. They are vulcanite rings made in pairs, in which one ring just fits into the other, so as to clip and stretch pieces of membrane between them. They will be found described and figured in *Journ. Roy. Mic. Soc.*, ii, 1879, p. 357, and in Robin's *Journ. de l'Anat.*, 1879, p. 54. They may be obtained, in sets of various sizes (that of seven eighths of an inch being the most convenient for 3 × 1 slides), of Burge and Warren, 42, Kirby Street, Hatton Garden, London, E.C., price ten shillings the dozen pairs.

This useful little apparatus has lately been re-invented by Eternod (*Zeit. f. wiss. Mik.*, iv, 1, 1887, p. 39), and is made according to his designs by Demaurex, Bandagiste, Fusterie, Geneva (Switzerland).

UNIVERSITY OF

and mounted on a slide in some suitable examination medium.

If the membrane were left in the water the cells would become detached, and would not be found in the finished preparation.

If the membrane had not been stretched as directed the silver would be precipitated not only in the intercellular spaces, but in all the small folds of the surface, and the forms of the cells would be disguised.

If the membrane had not been washed with distilled water before impregnation there would have been formed a deposit of silver on every spot on which a portion of an albuminate was present, and these deposits might easily be mistaken for a normal structure of the tissue. It is thus that very often impurities in the specimen have been described as stomata of the tissue.

If the solution be taken too weak—for instance, 1 : 500 or 1 : 1000, or if the light be not brilliant—a *general* instead of an *interstitial* stain will result; nuclei will be most stained, then protoplasm, and the intercellular substance will contain but very little silver.

In general, in a good "impregnation" the contents of cells, and especially nuclei, are quite invisible.

Ranvier notes that when tissues are to be impregnated by immersion they should be constantly *agitated* in the silver-bath in order to avoid the formation on their surfaces of deposits of chlorides and albuminates of silver, which would give rise to deceptive appearances.

Impregnation with silver may be followed by treatment with picro-carmine (or other carmine stain), which will bring out the nuclei, provided the impregnation has not been overdone.

It should be noticed that impregnations only succeed with *fresh* tissues, and cannot be made to succeed with tissues preserved in any way.

359. Silver Nitrate: the Solutions to be employed (Ranvier).—The solutions generally employed by Ranvier vary in strength from 1 : 300 to 1 : 500. Thus 1 : 300 is used for the epiploön, pulmonary endothelium, cartilage, tendon; whilst a strength of 1 : 500 is employed for the study of the phrenic centre, and for that of the epithelium of the intestine. For the impregnation of the endothelium of blood-vessels (by injection) solutions of 1 : 500 to 1 : 800 are taken.

M. Duval (*Précis*, p. 229) recommends solutions of 1, 2, or at most 3 per cent.

V. Recklinghausen used, for the cornea, a strength of from 1 : 400 to 1 : 500 (*Die Lymphgefässe*, &c., Berlin, 1862, p. 5).

Robinski (*Arch. de Physiol.*, 1869, p. 451) used solutions varying between 0·1 and 0·2 per cent., which he allowed to act for thirty seconds.

Reich (*Sitzb. d. wien. Akad.*, 1873, Abth. 3, April; *Zeit. f. wiss. Mik.*, i, p. 397) takes solutions of from 1 : 600 to 1 : 400 for the study of the endothelium of vessels by injection.

Rouget (*Arch. de Physiol.*, 1873, p. 603) employed solutions as weak as 1 : 750, or even 1 : 1000, exposing the tissues to their action several times over, and washing them with water after each bath.

The Hertwigs take, for marine animals, a 1 per cent. solution (*Jen. Zeit. f. Naturk.*, xvi, pp. 313 and 324).

The Hoggans (*Journ. of Anat. and Physiol.*, xv, 1881, p. 477) take, for lymphatics, a 1 per cent. solution.

Tourneux and Hermann (Robin's *Journal de l'Anat.*, 1876, p. 200), in their fine studies on the epithelia of Invertebrates, employed a solution of 3 : 1000 strength, and in some cases weaker solutions. The tissues were allowed to remain in the silver-bath for one hour, and were washed out with alcohol of 36° strength.

Hoyer (*Arch. f. mik. Anat.*, 1876, p. 649) takes a solution of nitrate of silver of known strength, and adds ammonia to it until the precipitate that is formed just redissolves, then dilutes the solution until it contains from 0·75 to 0·50 per cent. of the salt.

This *ammonio-nitrate* solution is intended principally for the impregnation of the endothelium of vessels by injection, but can also be used for the impregnation of membranes by pouring on. It has the advantage of impregnating absolutely nothing but endothelium or epithelium; connective tissue is not affected by it. It is also said to give a sharper localisation of the stain than the ordinary solutions.

Dekhuysen (*Anat. Anz.*, iv, 1889, No. 25, p. 789; *Zeit. f. wiss. Mik.*, vii, 3, 1890, p. 351) has applied to tissues of terrestrial animals the method of HARMER for marine animals (see below, § 363). He washes a portion of mesentery of a frog in a 1·3 per cent. solution of nitrate of potash, and brings it for from three to six minutes into a 0·25 per cent. solution of silver nitrate containing 3 per cent. of nitric acid. After that time it is brought into pure 3 per cent. nitric acid, thence after a few minutes into 96 per cent. alcohol, and then into clove oil, in which it is reduced in diffused light in a few minutes. The method is stated to have the advantage of giving an excellent fixation of tissues, and of allowing a good nuclear after-stain with hæmatoxylin, safranin, or methyl green.

REGAUD (*Journ. Anat. et Phys.*, xxx, 1894, p. 719; *Zeit. f. wiss. Mik.*, xii, 1, 1895, p. 74) recommends for the study of lymphatics a process devised by RENAUT. One per cent. solution of nitrate of silver is mixed either with three or four parts of a mixture of eighty parts of saturated solution of picric acid and twenty of 1 per cent. osmic acid solution, or with four parts of a mixture of forty parts of picric acid solution and twenty of osmic acid solution, with or without the addition of 1 per cent. of acetic acid to the mixture.

360. Other Salts of Silver.—ALFEROW (*Arch. de Physiol.*, 1874; *Laboratoire d'histologie du Collège de France*, 1874, p. 258; DUVAL, *Précis*, p. 230) recommends the soluble silver salts of organic acids, viz. the picrate, lactate, acetate, and citrate, as giving better results than the nitrate. He employs them in solutions of 1 : 800, and adds to the solution employed for staining a small quantity of the acid of the salt taken (10 to 15 drops of a concentrated solution of the acid to 800 c.c. of the solution of the salt). The object of the free acid is to decompose the precipitates formed by the action of the silver salt on the chlorides, carbonates, and other substances existing in the tissues, leaving only the albuminate, which is a more resistent compound.

361. Silver Nitrate: Reduction.—Reduction may be effected in other media than distilled water.

V. RECKLINGHAUSEN washed his preparations in salt solution before exposing them to the light in distilled water (*Arch. f. path. Anat.*, xix, p. 451). Physiological salt solution (0·75 per cent.) is commonly used for these washings.

MÜLLER (*Arch. f. path. Anat.*, xxxi, p. 110), after impregnation by immersion for two or three minutes in a 1 per cent. solution of nitrate of silver in the dark, adds to the solution a small quantity of 1 per cent. solution of iodide of silver (dissolved by the aid of a little iodide of potassium). After being agitated in this mixture the preparations are washed with distilled water, and exposed to the light for two days in a 1 per cent. solution of nitrate of silver (see also GIERKE, in *Zeit. f. wiss. Mik.*, i, 1884, p. 396).

ROUGET (*Arch. de Physiol.*, 1873, p. 603) reduces in glycerin.

SATTLER (*Arch. f. mik. Anat.*, xxi, p. 672) exposes to the light for a few minutes in water acidulated with acetic or formic acid. THANHOFFER (*Das Mikroskop*, 1880) recommends this method. He employs a 2 per cent. solution of acetic acid.

KRAUSE brings his preparations, after washings, into a light red solution of permanganate of potash. Reduction takes place very quickly, even in the dark. The method does not always succeed (see GIERKE, in *Zeit. f. wiss. Mik.*, i, 1884, p. 400).

OPPITZ brings his preparations for two or three minutes into a 0·25 or 0·50 per cent. solution of chloride of tin. Reduction takes place very rapidly (GIERKE, l. c.).

JAKIMOVITCH (*Journ. de l'Anat.*, xxiii, 1888, p. 142; *Journ. Roy. Mic. Soc.*, 1889, p. 297) brings nerve preparations, as soon as they have become of a dark brown colour, into a mixture of formic acid 1 part, amyl alcohol 1 part, and water 100 parts. The objects exposed to the light in this mixture for two or three days at first become brighter, a part of the reduced silver being dissolved; hence the mixture must be renewed from time to time. When all the silver has dissolved, a darker colour is permanently assumed. The nerve-cells are left in this mixture for five to seven days.

362. After-blackening.—LEGROS (*Journ. de l'Anat.*, 1868, p. 275) washes his preparations after reduction in hyposulphite of soda, which prevents after-blackening. According to DUVAL (*Précis*, p. 230) they should be washed for a few seconds only in 2 per cent. solution, and then in distilled water.

363. Silver Impregnation of Marine Animals.—On account of the considerable quantity of chlorides that bathe the tissues of marine animals, these cannot be treated *directly* with nitrate of silver.

HERTWIG (*Jen. Zeit.*, xiv, 1880, p. 324) recommends fixing them with a weak solution of osmic acid, then washing with distilled water until the wash-water gives no more than an insignificant precipitate with silver nitrate, and then treating for six minutes with 1 per cent. solution of silver nitrate.

HARMER (*Mitth. Zool. Stat. Neapel*, v, 1884, pp. 44 to 56) has discovered that many marine animals will live for some time (half an hour) in a 5 per cent. solution of nitrate of potash in distilled water. By washing them in this way they may be freed from the greater part of their chlorides, and may then be treated with silver nitrate in the usual way. This method gave good results with *Loxosoma* and *Pedicellina*, with Medusæ, Hydroids, *Sagitta* and *Appendicularia*.

VOSMAER has been able by this means to demonstrate the epithelium of *Chondrosia* and *Thenea*, which Sollas was unable to see; and MEYER has obtained good results with Annelids and ova of Teleostea. Few animals resist the action of nitrate of potash so well as *Loxosoma* and *Pedicellina*, but die in the solution in a few minutes. Their tissues, however, suffer but little change, and give good impregnations. Harmer thinks that for these animals other solutions having the same density as sea water might be substituted for the nitrate of potash, and recommends a 4·5 per cent. solution of sulphate of soda.

See also DEKHUYSEN's method, *ante*, § 359.

364. Impregnation of Nerve Tissue.—For this subject, which includes the important bichromate-and-silver method of GOLGI, see Part II.

365. Double-staining Silver-stained Tissues.—The nuclei of tissues impregnated with silver may be stained with the usual reagents, provided that solutions containing free ammonia be avoided, as this would dissolve out the silver. These stains will only succeed, however, with successful negative impregnations, as nuclei that have been impregnated will not take the second stain.

Impregnation with silver may be followed by impregnation with gold. In this case the gold generally substitutes itself for the silver in the tissues, and though the results are sharp and precise, the effect of a double stain is not produced.

Gold.

366. The Characters of Gold Impregnations.—Gold chloride differs from nitrate of silver in that it generally gives *positive* (§ 354) impregnations only. It only gives negative images, so far as I know, when caused to act on tissues that have first received a negative impregnation with silver, the gold substituting itself for the silver. In order to obtain these images you first impregnate very lightly with silver; reduce; treat for a few minutes with a 0·5 per cent. solution of gold chloride, and reduce in acidulated distilled water.

This process, however, is in but little use, and except for the staining of cytoplasm for cytological researches and for certain special studies on the cornea, and on connective tissue, the almost exclusive function of gold chloride is the impregnation of nervous tissue. For this tissue, gold chloride exhibits a remarkable selectivity, in virtue of which it justly ranks as a most valuable reagent for the study of nerve end-organs and the distribution of nerves.

For all the objects above named gold chloride is capable of furnishing preparations that for beauty and clearness cannot be surpassed, if even they can be equalled by any other means. A successful gold preparation shows at a glance, with diagrammatic clearness, a wealth of minute detail which perhaps can only be painfully glimpsed by other means. But not every gold preparation *is* successful. I think there is no use in blinking the fact that very few are successful (one of the most experienced authorities in the matter told me lately that, as to nerve end-organs at all events, one preparation in ten thousand is successful). I took up in the first edition of this work the doubtless unpopular position that "with all possible precautions gold chloride is uncertain in its action, and that the results obtained by means of it need to be controlled by the employment of other methods," and illustrated that position at considerable length.

That this position was the correct one is now generally admitted. It is acknowledged to be abundantly evident that the very best gold preparations give images that are only worthy of credence as to what they show, and furnish absolutely no evidence whatever as to the non-existence of anything that they do not show; for you can never be sure that the imbibition of the salt has not capriciously failed, or its reduction capriciously stopped at any point. That the images frequently *do* stop capriciously short in the representation of reality there is abundant evidence. One such case has been treated by me *ex professo* in *Recueil Zool. Suisse,* i, 1884, p. 685 (*Les organes chordotonaux des Diptères, et la méthode du chlorure d'or*).

The authors of some of the methods about to be described claim for them that they give premanent preparations. I warn the reader against indulging in the hope that, with all possible precautions, his preparations will retain all their beauty for more than a few weeks. A successful gold preparation is certainly a thing of beauty, but it is, unfortunately, by no means a joy for ever. The able histologist whose experience I have taken the liberty of quoting above tells me that "as to permanence, they are—

> 'Like the snowfall on the river.'"

Still, the greater the care taken in preparation, and particularly the greater the care taken to ensure thorough reduction of the gold, the longer will be the life of the preparations.

Careful attention to the devices to this end detailed in the following paragraphs will do much; and possibly LINDSAY JOHNSON's suggestion (*supra,* § 356) of the utility of "sunning" the solutions before use may prove an unexpected help.

367. As to the Commercial Salts of Gold.—It is necessary to remind the histologist that "all is not gold that glitters." Many things are not what they seem, and gold chloride is one of them, as will appear from the following quotation from SQUIRE's *Methods and Formulæ,* &c. (p. 43), an excellent authority on the chemistry of histological reagents:

"Commercial chloride of gold is not the pure chloride, $AuCl_3$, but the crystallised double chloride of gold and sodium, containing 50 per cent. of metallic gold.

"Commercial chloride of gold and sodium is the above crystallised double chloride mixed with an equal weight of chloride of sodium, and contains 25 per cent. of metallic gold."

368. The State of the Tissues to be impregnated.—Gold methods may be divided into two groups: the one, chiefly concerned with the study of peripheral nerves or nerve end-organs, is characterised by employing either *perfectly fresh* tissues or tissues that have been subjected to a special treatment by organic acids; the other, concerned with the study of nerve-centres, is characterised by the employment of tissues hardened in the usual way. This latter group naturally falls to be considered in Part II.

The hitherto classical rule, that for researches on nerve-endings the tissues should be taken perfectly fresh, seems not to be valid for all cases. For Drasch (*Sitzb. k. k. Akad. Wiss. Wien*, 1881, p. 171, and 1884, p. 516; and *Abhand. math.-phys. Cl. d. K. Sach. Ges. d. Wiss.*, xiv, No. 5, 1887; *Zeit. f. wiss. Mik.*, iv, 4, 1887, p. 492) finds that better results are obtained with tissues that have been allowed to lie after death for twelve, twenty-four, or even forty-eight hours in a cool place. He even suspects that the function of the organic acids in the methods inspired by Löwit's method is to bring the tissues into somewhat the state in which they are naturally found at a certain moment of post-mortem process—a state, namely, in which the nerves have a special susceptibility for impregnation with gold.

369. Cohnheim's **Method** (*Virchow's Arch.*, Bd. xxxviii, pp. 346—349; *Stricker's Handb.*, p. 1100).—This, the archetype of the gold methods, was as follows:—Fresh pieces of cornea (or other tissue to be operated upon) are put into solution of chloride of gold of 0·5 per cent. strength until they are thoroughly yellow, and then exposed to the light in water acidulated with acetic acid until the gold is thoroughly reduced, which happens in the course of a few days at latest. They are then mounted in acidulated glycerin.

The method in this, its primitive form, often gave splendid results, but was very uncertain, giving sometimes a nuclear or protoplasmic stain, sometimes an extra-cellular impregnation similar to that of nitrate of silver. And the preparations thus obtained are anything but permanent.

370. Löwit's **Method.**—The principle of this process is that, in order to facilitate the penetration of the gold and its subsequent reduction in the tissues, the tissues are made to swell up by treatment with formic acid before being brought into the gold-bath, and formic acid is employed to assist the reduction after impregnation.

The following directions as to this method, which may serve as a type of the modern methods of research on nerve-endings, are taken from FISCHER's paper on the corpuscles of Meissner (*Arch. f. mik. Anat.*, xii, 1875, p. 366).

Löwit's method was first published by him in the *Wien. Sitzgsber.*, Bd. lxxi, Abth. 3, 1875, p. 1.

Small pieces of *fresh* skin are put into dilute formic acid (one volume of water to one of the acid of 1·12 sp. gr.), and remain there until the epidermis peels off. They then are put for fifteen minutes into gold chloride solution (1½ per cent. to 1 per cent.), then for twenty-four hours into dilute formic acid (1 part of the acid to 1—3 of water), and then for twenty-four hours into undiluted formic acid. (Both of these stages are gone through in the dark.) Thin sections are then made and mounted in dammar or glycerin. Successful preparations show the nerves alone stained, but it is not possible always to control the results.

371. RANVIER'S **Formic Acid Method** (*Quart. Journ. Mic. Sci.* [N.S.], lxxx, 1880, p. 456).—The method of Löwit has been modified by many workers by omitting the final treatment with undiluted formic acid, and also in some other details. Ranvier proceeds as follows :—Reflecting that the action of the one third formic acid in which Löwit placed his tissues must be hurtful to the final ramifications of the nerves, he combines the formic acid with a fixing agent designed to antagonise its altering action, and takes for this purpose the chloride of gold itself. The tissues are placed in a mixture of chloride of gold and formic acid (4 parts of 1 per cent. gold chloride to 1 part of formic acid) which has been boiled and allowed to cool (Ranvier's *Traité*, p. 826). They remain in this until thoroughly impregnated (muscle twenty minutes, epidermis two to four hours); the reduction of the gold is effected either by the action of daylight in acidulated water, or in the dark in dilute formic acid (1 part of the acid to 4 parts of water).

The object of boiling the mixture of gold chloride and formic acid is this, that "by boiling in the presence of the acid the gold acquires a great tendency to reduction, and for this reason its selective action on nervous tissues is enhanced."

372. Ranvier's **Lemon-juice Method** (*Traité,* p. 813).—Instead of combining the formic acid with gold chloride in order to mitigate its action, recourse may be had to a less injurious acid than formic acid. Ranvier finds that of all acids lemon-juice is the least hurtful to nerve-endings. He therefore soaks pieces of tissue in fresh lemon-juice, filtered through flannel, until they become transparent (five or ten minutes in the case of muscle). They are then rapidly washed in water, brought for about twenty minutes into 1 per cent. gold chloride solution, washed again in water, and brought into a bottle containing 50 c.c. of distilled water and two drops of acetic acid. They are exposed to the light, and the reduction is complete in twenty-four to forty-eight hours. The preparations thus obtained are good for immediate study, but are not permanent on account of their over-blackening with time, the reduction of the gold being incomplete. In order to obtain perfectly reduced, and therefore permanent, preparations, the reduction should be done in the dark in a few cubic centimetres of dilute formic acid (1 part acid to 4 of water). The reduction is complete in twenty-four hours.

373. Viallanes' **Osmic Acid Method** (*Hist. et dev. des Insectes,* 1883, p. 42).—The tissues are treated with osmic acid (1 per cent. solution) until they begin to turn brown, then with one fourth formic acid for ten minutes; they are then put into solution of chloride of gold of 1 : 5000 (or even much weaker) for twenty-four hours in the dark, then reduced in the light in one fourth formic acid. According to my experience this is a very excellent method, both the fixation by osmic acid and the great dilution of the gold solution being features likely to be of advantage in many cases.

374. Other Methods.—The numerous other methods that have been proposed differ from the foregoing partly in respect of the solutions used for impregnation, but chiefly in respect of details imagined for the purpose of *facilitating the reduction* of the gold, and rendering it as complete as possible.

Thus BASTIAN modified Cohnheim's original method by employing a solution of gold chloride of a strength of 1 to 2000, acidulated with HCl (1 drop to 75 c.c.), and performing the reduction in a mixture of equal parts of formic acid and water *kept warm,* heat being an agent that furthers reduction.

HÉNOCQUE (*Arch. de l'Anat. et de la Physiol.,* 1870, p. 111) impregnates in a 0·5 per cent. solution of gold chloride, washes in water for twelve to twenty-four hours, and reduces, with the aid of heat, in a nearly saturated solution of tartaric acid. The tartaric acid solution must be contained in a well-stoppered bottle. The best temperature for reduction is 40° to 50° C. Reduction is effected very rapidly, sometimes in a quarter of an hour.

This process has been described as the method of CHRSCHTSCHONOWIC (*Arch. f. mik. Anat.,* vii, 1872, p. 383).

HOYER (*Arch. f. mik. Anat.,* ix, 1873, p. 222) proceeds as follows :—(For corneal nerves.) The double chloride of gold and potassium has the following advantages over the simple gold chloride. It is more easy to be obtained of unvarying composition, it is more perfectly neutral, and its solutions are more perfectly stable. It is used in solutions of the same strength as chloride of gold, viz. 0·5 per cent. Corneæ must be very thoroughly imbibed with the solution. Small corneæ (rabbit, guinea-pig) require half to one hour, human corneæ two to five hours (in an acidulated solution). It is better to err on the side of too prolonged immersion rather than the contrary. In order to demonstrate the intra-epithelial ramifications of nerves, the gold is partially reduced by exposure for sixteen to twenty-four hours in (one or two ounces of) distilled water, and there is added to the water one or two drops of a pyrogallic acid developing solution, such as is used in photography (*vide* GERLACH, *Die Photographie als Hülfsmittel der mikroskopischen Forschung,* Leipzig, 1863). Or instead of treating them with the developing solution, the corneæ may be removed to a warm concentrated solution of tartaric acid, and remain there at the temperature of an incubating stove until the gold is fully reduced.

I have myself used the double chloride of gold and sodium with good results.

Ciaccio (*Journ. de Microgr.*, vii, 1883, p. 38; *Journ. Roy. Mic. Soc.* [N.S.], iii, 1883, p. 290) prefers the double chloride of gold and cadmium.

For Gerlach's method for hardened nerve-centres see Stricker's *Handb.*, 1872, p. 678. It may be considered to be now superseded by Golgi's bichromate of silver process.

Flechsig (*Die Leitungsbahnen im Gehirn*, 1876; *Arch. f. Anat. u. Phys.*, 1884, p. 453) reduces in a 10 per cent. solution of caustic soda.

Nesteroffsky treats impregnated preparations with a drop of sulphydrate of ammonium, and finishes the reduction in glycerin (quoted from Gierke's *Färberei z. mik. Zwecken*).

Böhm reduces in Pritchard's solution.

Pritchard's solution consists of amyl alcohol, 1 per cent.; formic acid, 1 per cent.; and water, 98 per cent.

Manfredi treats fresh tissues as follows (*Arch. per le Sci. med.*, v, No. 15):—Gold chloride, 1 per cent., half an hour; oxalic acid, 0·5 per cent.; they are then warmed in a water-bath to 36°, allowed to cool, and examined. Mount in glycerin. Sunny weather is necessary.

He treats tissues previously hardened in 2 per cent. solution of bichromate of potash, as follows (*ibid.*). They are put for half an hour into solution of arsenic acid, or into 1 per cent. acetic acid. They are then put into 1 per cent. gold chloride for half an hour, washed in water, and reduced in sunlight in 1 per cent. arsenic acid solution, which is changed for fresh as fast as it becomes brown. Mount in glycerin. Sunny weather is necessary.

Boccardi (*Lavori Instit. Fisiol. Napoli*, 1886, i, p. 27; *Journ. Roy. Mic. Soc.*, 1888, p. 155) recommends oxalic acid of 0·1 per cent. or of 0·25 to 0·3 per cent., or a mixture of 5 c.c. pure formic acid, 1 c.c. of 1 per cent. oxalic acid, and 25 c.c. of water. Objects should remain in this fluid in the dark not longer than two to four hours.

Kolossow (*Zeit. f. wiss. Mik.*, v, 1, 1888, p. 52) impregnates for two or three hours in a 1 per cent. solution of gold chloride acidulated with 1 per cent. of HCl, and reduces for two or three days in the dark in a 0·01 per cent. to 0·02 per cent. solution of chromic acid.

Underwood (*Journ. Brit. Dental Ass.*, xi, 1890, p. 696; *Journ. Roy. Mic. Soc.*, 1890, p. 815) gives the following:—Wash sections in bicarbonate of soda (strength not given); treat them for half an hour to an hour with 1 per cent. solution of chloride of gold, neutralised if acid by bicarbonate of soda; wash in water; reduce until the sections turn crimson (about an hour) in 1 per cent. formic acid kept "fairly hot" on a water-bath, in the dark; wash for half an hour in cold water; dry the sections (*sic*) and mount them in glycerin jelly ("specimens mounted in balsam always go wrong"). This is for sections of decalcified teeth.

Geberg (*Intern. Monatsschr.*, x, 1893, p. 205) states that previous treatment of tissues for twenty-four hours with lime-water (Arnstein's method) greatly helps the reduction.

Bernheim (*Arch. f. Anat. u. Phys., Phys. Abth.*, 1892, Supp., p. 29; *Zeit. f. wiss. Mik.*, x, 4, 1893, p. 484) adds to Löwit's dilute formic acid a piece of sulphite of sodium (must be fresh and smell strongly of sulphurous acid).

Dr. Lindsay Johnson writes to me that besides the "sunning" of the impregnating solution recommended above (§ 356), the following precautions should be taken:—"The tissue must be well washed in distilled water, and the gold carefully acidulated with a neutral acetate or formiate, or acetic or formic acid, at least twenty-four hours before using; and then afterwards the tissue must be washed until no reaction occurs to test-paper."

For the details of the application of the methods of which the principles have been set forth above, and for those of the important processes of impregnation of central nerve organs, the reader is referred to those chapters of Part II which treat of nerve-tissues and organs.

375. Ulterior Treatment of Impregnated Preparations.—Preparations may be mounted either in balsam or in acidulated glycerin (1 per cent. formic acid).

Theoretically they ought to be permanent if the reduction of the metal has been completely effected.

In practice, all are doomed to destruction in course of time by after-blackening, and few will be found to survive more than a few months. Ranvier states that this can be avoided by putting the preparations for a few days into alcohol, which possesses the property of stopping the reduction of the gold. But this must be taken to mean that by this device the period of usefulness of the preparations may be prolonged for some time, not indefinitely.

Blackened preparations may be *bleached* with cyanide or ferrocyanide of potassium. REDDING employs a weak solution of ferrocyanide; CYBULSKY a 0·5 per cent. solution of cyanide. But the results are far from being perfectly satisfactory.

Preparations may be double-stained with the usual stains (safranin and methyl green being very much to be recommended), but nuclei will only take the second stain in the case of negative impregnation.

376. Impregnation of Marine Animals.—For some reason that I am unable to explain, the tissues of marine animals do not readily impregnate with gold in the fresh state. It is said by FOL that impregnation succeeds better with spirit specimens.

Other Metallic Stains.

377. Osmic Acid and Pyrogallol.—Everybody knows that osmic acid stains tissues. Most people, I should think, would be heartily glad if it did not. Meanwhile, to make the best of this willy-nilly stain, you may sometimes find it useful to treat the tissues with weak pyrogallic acid, which will very quickly turn them of a fine greenish black, sometimes giving useful differentiations.

This method was first published by me in 1887 (*La Cellule*, t. iv, fasc. 1, p. 110), and I have since republished it twice, with qualified recommendation. I have found it occasionally very useful, but have hesitated to recommend it for general work because I have usually found the stain too energetic and too little discriminating. It frequently happens that everything in a preparation comes out blacker than ink.

At the time of the publications mentioned above I had only tried the reaction with tissues impregnated with *pure* osmic acid. Since then HERMANN (*Arch. f. mik. Anat.*, xxxvii, 4, 1891, p. 570) tried it with tissues fixed in his platino-aceto-osmic mixture (§ 65). With this modification of the process incomparably better results are obtained, as I can testify.

HERMANN's procedure is as follows:—The tissues are put for one or two days into the platino-aceto-osmic mixture, washed thoroughly in water, and hardened in successive alcohols; after which, to obtain the black reaction, they are put for twelve to eighteen hours into raw pyroligneous acid. This acid ought (*Ergebnisse der Anat.*, ii, 1893, p. 28) to be as raw as possible, and to be of a dark brown colour and evil-smelling.

According to my experience, the particular procedure of HERMANN is not that which gives the best results. I now proceed as follows:

Either the mixture of HERMANN or the mixture of FLEMMING may be used for fixing. You may leave the tissues therein for twelve or twenty-four hours if you think that that is desirable in the interest of a complete fixation; but in the interest of the stain alone, half an hour is enough and *is preferable.* It is not only useless but hurtful to put the preparations into alcohol after fixation, for it is desirable that the tissue should be in as *fresh* a state as possible on coming into the pyrogallol. In consequence it is not possible to obtain the best results by treating paraffin sections. The tissues are therefore brought in bulk, directly after fixing, into pyrogallol. You may use Hermann's pyroligneous acid if you like, but I consider that a weak solution of pyrogallol is preferable. In pyrogallol it seems to me that a finer differentiation is obtained, and that the preservation of the tissues is sensibly better. The tissues may remain in either of these liquids for twenty-four hours, but for small objects an hour or less is sufficient. An alcoholic solution of pyrogallol *may* be taken if desired, and this *may* be indicated in certain cases. I have not obtained the reaction with tannin used on chrom-osmium material.

The method as thus modified is important. There is obtained a black stain, which is at the same time a plasma stain and a nuclear stain, chromatin being so far stained that it is not necessary to have recourse afterwards to a special chromatin stain. This is one of the best methods that I know of for the study of Nebenkerne. With Invertebrates it sometimes gives very elegant differentiations of nervous tissue. It is a *very easy* method, and if pyrogallol be used a very *safe* one (with pyroligneous acid not so safe). But it is not quite a first-class method, for the plasma stain obtained is not quite of the highest order, the cytoplasmic hyaloplasm or enchylema being too frequently stained at the same time as the reticulum.

Although, as said, this method enables one to dispense with a special second chromatin stain, yet it is frequently very advantageous to use one. I greatly recommend safranin (stain very strongly, twenty-four hours at least, and start the extraction with acid alcohol).

This method has been attributed to VON MAEHRENTHAL. A modification of this method is said by AZOULAY to give a specific stain of the medullary sheath of nerves, see his process under "Neurological Methods" in Part II. See also a similar process for medullated nerve by HELLER and GUMPERTZ, quoted *Zeit. f. wiss. Mik.*, xii, 3, 1896, p. 385.

The communications of KOLOSSOW (*Zeit. f. wiss. Mik.* ix, 1, 1892, p. 38, and ix, 3, 1893, p. 316) do not appear to me to constitute a useful contribution to the subject.

378. The method of BRÖSICKE (*Centralb. f. d. med. Wiss.*, 1879, p. 873; *Zeit. f. wiss. Mik.*, i, 1884, p. 409) consists in treating osmium objects for twenty-four hours with a solution of one part of oxalic acid in fifteen parts of water. This gives a Burgundy-red stain. It is necessary that the objects be washed and brought into the oxalic acid as soon as possible after the treatment with osmium, as if the osmium has once begun to blacken them, the oxalic acid is powerless afterwards to redden them. I consider this method to be superseded by the pyrogallol process.

379. Perchloride of Iron.—This reagent, introduced by POLAILLON (*Journ. de l'Anat.*, iii, 1866, p. 43), sometimes gives most useful results, especially in the study of peripheral nerve-ganglia, in which it stains the nervous tissue alone, the connective tissue remaining colourless. The method consists in impregnating in perchloride of iron, and reducing in tannic, gallic, or pyrogallic acid.

The HOGGANS, who have done very good work with this reagent, proceed as follows (*Journ. Quekett Club*, 1876; *Journ. Roy. Mic. Soc.*, ii, 1879, p. 358):—The tissue (having been first fixed with silver nitrate, which is somewhat reduced by a short exposure to diffused light) is dehydrated in alcohol, and treated for a few minutes with 2 per cent. solution of perchloride of iron in spirit. It is then treated with a 2 per cent. solution of pyrogallic acid in spirit, and in a few minutes more, according to the depth of tint required, may be washed in water and mounted in glycerin.

FOL (see *ante*, § 67) fixes in perchloride solution, and treats the preparations for twenty-four hours with alcohol containing a trace of gallic acid.

POLAILLON (*l. c.*) reduces in tannic acid.

This method is not applicable to chromic objects.

I should add that in my own experience I have found it very useful in certain special cases.

380. Pyrogallate of Iron (ROOSEVELT, *Med. Rec.*, ii, 1817, p. 84; *Journ. Roy. Mic. Soc.*, 1888, p. 157).—A stain compound of 20 drops of saturated solution of iron sulphate, 30 grms. water, and 15 to 20 drops pyrogallic acid.

381. **Palladium Chloride** (F. Schultze, see *ante*, §§ 66 and 102). **Prussian Blue** (see Leber, *Arch. f. Ophthalm.*, xiv, p. 300; Ranvier, *Traité*, p. 108). **Cupric Sulphate** (see Leber, *ibid.*). **Lead Chromate** (see Leber, *ibid.*). **Sulphides** (see Landois, *Centralb. f. d. med. Wiss.*, 1885, No. 55; and Gierke, in *Zeit. f. wiss. Mik.*, i, 1884, p. 497). **Molybdate of Ammonium** (Merkel; Krause) (see Gierke, *Zeit. f. wiss. Mik.*, i, 1884, p. 96). **Oxychloride of Ruthenium** (Nicolle and Cantacuzène) (see *Ann. Inst. Pasteur*, vii, 1893, p. 331). **Impregnation with Fats, Altmann's Method** (see *post*, "Corrosion").

CHAPTER XXI.

EXAMINATION AND PRESERVATION MEDIA.

382. Introductory.—I comprehend under this heading all the media in which an object may be examined. The old distinction of "indifferent" liquids, and those which have some action on tissues, appears to be misleading more than helpful; inasmuch as it is now well understood that *no* medium is without action on tissues except the plasma with which they are surrounded during the life of the organism; and this plasma itself is only "indifferent" whilst all is *in situ;* as soon as a portion of tissue is dissected out and transferred to a slide in a portion of plasma the conditions become evidently artificial.

It does not appear necessary to create a separate group for mounting media, as all preservative media may be used for mounting.

For directions as to making permanent mounts in fluid media see the early sections of Chap. XXII.

383. Water.—To preserve it from mould, a lump of thymol or camphor should be kept in the supply. Water may be employed without inconvenience, and sometimes (on account of its low index of refraction) with great advantage for the examination of all structures that have been fixed with osmic or chromic acid, or some salt of the heavy metals; but it is by no means applicable to the examination of fresh tissues,—that is, tissues that have not been so fixed. It is important that the beginner should bear in mind that water is very far from being an "indifferent" liquid; many tissue-elements are greatly changed by it (nerve-end structures, for instance), and some are totally destroyed by its action if prolonged (for instance, red blood-corpuscles).

384. Theory of Indifferent Liquids.—In order to render water inoffensive to such tissues as these it must, firstly, have dissolved in it some substance that will give it a density equal to that of the liquids of the tissue, so as to prevent the occurrence of osmosis, to which process the destructive action of pure water is mainly due. Salt solution is a medium suggested by this necessity. But salt solution by no means fulfils all the conditions implied in the notion of an "indifferent" liquid. In so far as it possesses a density approaching to that of the liquids of the tissues, one cause of osmosis is eliminated; but there remains another, due to the difference of composition of the liquids within the tissues and that without. Cell contents are a mixture of colloids and crystalloids; salt solution contains only a crystalloid, whose high diffusibility causes it to diffuse over into the colloids of the tissues. In order to reduce the consequent osmotic processes to a minimum, it is necessary that the examination medium contain, in addition to a due proportion of salt or other crystalloid, also a due proportion of colloids. By adding, for instance, white of egg to salt solution this end may be attained; and, as a matter of fact, the liquids recommended as indifferent are found invariably to contain both crystalloids and colloids. Thus (as stated by Frey) vitreous humour contains 987 parts of water to about 4·6 of colloid matters and 7·8 of crystalloids (common salt). In 1000 parts of liquor amnii are contained about 3·8 parts of colloid matter (albumen), 5·8 of salt, and 3·4 of urea. In blood-serum, 8·5 of colloids and 1 of crystalloid substance are found.

385. Salt Solution ("normal salt solution," "physiological salt solution").—0·75 per cent. sodium chloride in water. Carnoy recommends the addition of a trace of osmicacid.

386. Pictet's **Liquid** (*Mitth. Zool. Stat. Neapel,* x, 1, 1891, p. 89).—Five to ten per cent. solution of chloride of manganese. According to my experience, this solution is excellent, and very often advantageously takes the place of "normal salt solution." The proportions given are for *marine* animals, and for terrestrial animals will generally be found much too high. For these from 1 per cent. to 3 per cent. will be nearer the mark.

387. Iodised Serum.—Iodised serum was first recommended by Max Schultze (*Virchow's Archiv,* xxx, 1864, p. 263). I take the following instructions concerning it from Ranvier (*Traité,* p. 76).

The only serum that gives really good results is the amniotic liquid of mammals. A gravid uterus of a sheep or cow having been obtained (in large slaughterhouses such can be obtained without difficulty), an incision is made through the wall of the uterus and the fœtal membranes. A jet of serum issues from the incision, and is caught in a flask prepared for the purpose. Flakes of iodine are then added, and the flask is frequently agitated for some days. Two points should be noted. A perfectly fresh amnios must be taken, for the merest incipience of putrefaction will spoil the preparation. The flask should have a wide bottom, so that the serum may form only a shallow layer in it; otherwise the upper layers will not be sufficiently exposed to the action of the iodine.

Another method is as follows:—Serum is mixed with a large proportion of tincture of iodine; the precipitate of iodine that forms is removed by filtration, and there remains a strong solution of iodine in serum. This should be kept in stock, and a little of it added every two or three days to the serum that is intended for use.

Ranvier explains that at the outset serum dissolves very little iodine; but if an excess of iodine be kept constantly present in the solution, it will be found that after two or three weeks iodides are formed, and allow fresh quantities of iodine to dissolve; so that after one or two months a very strongly iodised serum is obtained. It should be dark brown. Such a solution is the most fitting for the purpose of iodising fresh serum in the manner directed above, and for making the different strengths of iodised serum that are required for different purposes. In general, for maceration purposes, a serum of a pale brown colour should be employed.

388. Aqueous Humour, Simple White of Egg.—Require no preparation beyond filtering. They may be iodised if desired.

389. Artificial Iodised Serum (Frey, *Le Microscope*, p. 131).

Distilled water	135 grms.
White of egg	15 ,,
Sodium chloride	0·20 grm.
Mix, filter, and add—	
Tincture of iodine	3 grms.

There is formed a precipitate, which is removed by filtering through flannel; and a little iodine is added to the filtrate.

390. KRONECKER'S **Artificial Serum** (from VOGT et YUNG, *Traité d'Anat. comp. prat.*, p. 473: I have been unable to discover the original source).

Common salt	6 grms.
Caustic soda	0·06 grm.
Distilled water	1000 grms.

391. MIGULA'S **Glycerized Blood-serum** (see the paper in *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 172; also *Journ. Roy. Mic. Soc.*, 1890, p. 804).

392. Syrup.—An excellent medium for examining many structures in the fresh state. To preserve it from mould, chloral hydrate may conveniently be dissolved in it (1 to 5 per cent.). I have used as much as 7 per cent., and found no disadvantage.

Carbolised Syrup.—Carbolic acid may be employed instead of chloral; 1 per cent. is sufficient.

Either of these syrups may be used as a mounting medium, but they are not to be recommended for that purpose, as there is always risk of the sugar crystallising out.

A good strength for syrup is equal parts of loaf sugar and water. Dissolve by boiling.

393. Acetate of Alumina (GANNAL'S **Solution**, BEALE, *How to Work*, &c., p. 58).

Acetate of alumina	1 part.
Water	10 parts.

394. Acetate of Potash (MAX SCHULTZE, *Arch. mik. Anat.*, vii, 1872, p. 180).—A nearly saturated solution in water. It is used by letting a drop run in under the cover-glass to the object, which is in water. After twenty-four hours the mount may be closed. The index of refraction is lower than that of glycerin.

This medium has been frequently recommended as having the property of preventing the blackening of objects that have been treated with osmium; but it seems extremely doubtful whether this is really the case.

395. Chloral Hydrate.—5 per cent. in water (LADOWSKY, *Arch. f. mik. Anat.*, 1876, p. 359).

Or, 2·5 per cent. in water (Brady, *British Copepods*).

Or, 1 per cent. in water (Munson, *Journ. Roy. Mic. Soc.*, 1881, p. 847).

396. Alcohol.—Not very recommendable for mounting, as if taken weak it is not a very efficient preservative, and if taken strong it attacks the cement of mounts.

Carpenter (*The Microscope*) recommends a strength of 1 part to 5 of water.

The chief use of alcohol for preservation purposes is of course for preserving specimens in till wanted for further preparation and study. See, on this point, the remarks in Chap. I, § 3.

397. Formaldehyde. See §§ 86 and 109.

Mercurial Liquids.

398. Gilson's **Fluid** (Carnoy's *Biologie cellulaire*, p. 94).

Alcohol of 60 per cent. . . .	60 c.c.
Water	30 „
Glycerin	30 „
Acetic acid (15 parts of the glacial to 85 of water)	2 „
Bichloride	0·15 grm.

A really excellent medium for the study of fine cellular detail with well-fixed objects.

399. Gage's **Albumen Fluid** (*Zeit. f. wiss. Mik.*, 1886, p. 223).

White of egg	15 c.c.
Water	200 „
Corrosive sublimate	0·5 grm.
Salt	4 grms.

Mix, agitate, filter, and preserve in a cool place. Recommended for the study of red blood-corpuscles and ciliated cells.

400. Pacini's **Fluids** (*Journ. de Mic.*, iv, 1880; *Journ. Roy. Mic. Soc.* [N.S.], ii, 1882, p. 702, and previous editions of this work).—These antiquated formulæ are quite superfluous for the study of fixed tissues. They consist essentially of corrosive sublimate of from one half to one third per cent. strength, with the addition of a little salt or acetic acid.

401. Harting's **Fluid.**—See *Micro. Dict.*, art. "Preservation," p. 640.

402. Goadby's **Fluids** (*Micro. Dict.*, art. "Preservation," or previous editions of this work).—They are quite unsuited for histological purposes.

403. Owen's **Fluid** (see Vogt et Yung, *Traité d'Anat. comp. pratique*, p. 19, or previous editions of this work).—It is quite superfluous for tissues that have been duly fixed.

Other Fluids.

404. Chloride and Acetate of Copper (Ripart et Petit's fluid, *Brebissonia*, 1880, p. 92; Carnoy's *Biol. cell.*, p. 95).

Camphor water (not saturated)	75 grms.
Distilled water	75 ,,
Crystallised acetic acid	1 grm.
Acetate of copper	0·30 ,,
Chloride of copper	0·30 ,,

This is certainly a *most valuable* medium for work with delicate fresh tissues. It may be used in combination with methyl green, *which it does not precipitate.* The most delicate elements are perfectly preserved in it; the addition of a drop of osmic acid or corrosive sublimate does not cause the least turbidity, and enhances its *fixing* action.

405. Tannin (Carnoy, *l. c.*).

Water	100 grms.
Powdered tannin	0·50 grm.

406. Picro-carmine.—Picro-carmine has been recommended by Ranvier as a medium for teasing fresh tissues in, in the belief that it possesses sufficient fixing action to preserve the form of cells. Carnoy finds that cells live in it for a considerable time, and become gorged with water and deteriorated to a considerable degree. Unfortunately, too, picro-carmine cannot be combined with a good fixing agent, as it is precipitated by alcohol and by acids, and especially by osmic acid.

407. Methyl Green.—See under Staining Agents. The aqueous solution is very useful as an examination medium for fresh tissues. It should be taken fairly concentrated, in which state it has sufficient fixing power, which is enhanced by the addition of a trace of osmic acid.

408. Wickersheimer's **Fluid** (*Zool. Anz.*, 1879, p. 670; cf. *Journ. Roy. Mic. Soc.*, 1882, p. 427; *id.*, 1880, p. 355; and *Entomol. Nachr.* 1880, p. 129).—This once famous fluid appears to be quite unsuccessful for histological purposes.

409. Meyer's **Salicylic Vinegar Preservative Solutions** (*Arch. mik. Anat.*, xiii, 1876, p. 868).—"Salicylic vinegar" is a solution of 1 part of salicylic acid in 100 parts of pyroligneous acid. The pyroligneous acid should be of 1·04 specific gravity, and should be of a pale yellow colour. This product is found in commerce, and may be obtained from Herrn J. M. Andreæ, Droguerie-Handlung, Frankfurt-a.-M.

1ST FLUID:

One vol. salicylic vinegar to 10 vols. of the following dilute glycerin, viz. glycerin 1 vol., water 2 vols.

For various larvæ, Hydræ, Nematodes, &c.

2ND FLUID:

One vol. salicylic vinegar to 10 vols. of the following dilute glycerin, viz. glycerin 1 vol., water 4 vols.

For Infusoria.

410. NOLL'S **Salicylic Vinegar and Gum Medium** (*Zool. Anz.*, 1883, p. 472).—A mixture of equal vols. of Meyer's second fluid (*ante*, last formula) and Farrant's medium (*post*, § 413.)

This mixture never becomes turbid, and does not dry up. The covers may be luted with asphalt or any other cement. The fluid answers admirably for delicate Crustacea and their larvæ; the preparations do not shrink, and are not too much cleared. It also answers well for hardened and stained preparations of Hydroids, small Medusæ, and other Cœlenterates.

411. DEANE'S **Medium** (see *Micro. Dict.*, art. " Preservation ").—Appears to be now superfluous.

412. HOYER'S **Gum with Chloral Hydrate or Acetate of Potash** (*Biol. Centralb.*, ii, 1882, pp. 23–4; *Journ. Roy. Mic. Soc.* [N.S.], iii, 1883, pp. 144–5).—A high 60 c.c. glass with a wide neck is filled two thirds full with gum arabic (in pieces), and then *either* a solution of chloral (of several per cent.) containing 5—10 per cent. of glycerin is added, *or* acetate of potash or ammonia. The gum with frequent shaking dissolves in a few days, and forms a syrupy fluid, which is slowly filtered for twenty-four hours. The clear filtered fluid will keep a long time, but if spores of fungi begin to develop a little chloral can be added and the fluid refiltered. The solution with chloral is for carmine or hæmatoxylin objects—that with acetate for anilin objects.

413. FARRANT'S **Medium** (BEALE, *How to Work*, &c., p. 58).

Picked gum arabic	4 ounces.
Water	4 ,,
Glycerin	2 ,,

To be kept in a stoppered bottle with a lump of camphor.

This medium is quoted by Frey as consisting of equal parts of gum, glycerin, and saturated aqueous solution of arsenious acid.

The *Micrographic Dictionary* gives the following directions: —Gum arabic 1 ounce, glycerin 1 ounce, water 1 ounce, arsenious acid 1½ grains; dissolve the arsenious acid in the water, then the gum (without heat), add the glycerin, and incorporate with great care to avoid forming bubbles.

Another method for making this medium is given by A. F. Stanley Kent in *Journ. Roy. Mic. Soc.*, 1890, p. 820.

414. Gum and Glycerin Medium (Langerhans' formula, a modification of Farrant's medium, *Zool. Anzeig.*, ii, 1879, p. 575).

Gummi arab.	5·0
Aquæ	5·0

To which after twelve hours are added—

Glycerini	5·0
Sol. aquosa acid. carbol. (5·100) . .	10·0

Marine animals may be preserved in this by simply running in a drop under the cover, and next day or later adding what is necessary to make up for evaporation, and closing the mount. Shrinkage is very slight, and most colours keep well.

415. Faris's **Glycero-gum** (*The Microscope*, x, 1890, p. 59; *Journ. Roy. Mic. Soc.*, 1890, p. 414).—Gum arabic 2 ounces, glycerin 1·5 ounces, water 1·5 ounces, thymol 1 grm. Mix, dissolve with heat, and filter.

416. Gum and Glycerin Jelly (Shimer, *The Microscope*, ix, 1889, p. 138; *Journ. Roy. Mic. Soc.*, 1890, p. 411).—Equal parts of glycerin jelly (Fol's second formula, *post*, § 432), Farrant's solution, and glycerin.

417. Cole's **Gum and Syrup Medium.** See § 179.

418. Apáthy's **Gum and Syrup Medium** (see Chap. IX, § 293).—This medium is recommended by Apáthy in a general way, and not merely for the special purpose for which it is quoted in § 293. It sets very hard, and, combined with a paper cell (see § 453), may be used for ringing glycerin mounts.

419. Fabre-Domergue's **Glucose Medium** (*La Nature,* No. 823, 9, Mars, 1889, supp.).

Glucose syrup diluted to twenty-five degrees of the areometer (sp. gr. 1·1968) . . .	1000 parts.
Methyl alcohol	200 ,,
Glycerin	100 ,,
Camphor, to saturation.	

The glucose is to be dissolved in warm water, and the other ingredients added. The mixture, which is always acid, must be neutralised by the addition of a little potash or soda.

This medium is said to preserve without change almost all animal pigments.

420. Brun's **Glucose Medium** (from Fabre-Domergue's *Premiers Principes du Microscope et de la Technique microscopique,* Paris, 1889, p. 123).

Distilled water	140 parts.
Camphorated spirit . . .	10 ,,
Glucose	40 ,,
Glycerin	10 ,,

Mix the water, glucose, and glycerin, then add the spirit, and filter to remove the excess of camphor which is precipitated on mixing. I am indebted to Dr. Henneguy for calling my attention to this liquid, which is an important one. It is preferable to glycerin because it preserves the colour of preparations stained with anilin dyes, *methyl green included.*

421. Levulose as a Mounting Medium.—Levulose is recommended as a mounting medium by Behrens, Kossel, u. Schiefferdecker (*Das Mikroskop u. d. Meth. d. mik. Unters.,* Braunschweig, 1889). It is uncrystallisable, and preserves well carmine and coal-tar stains (hæmatoxylin stains fade somewhat in it). The index of refraction is somewhat higher than that of glycerin. Objects may be brought into it out of water.

Glycerin Media.

422. Glycerin.—Glycerin diluted with water is frequently employed as an examination and mounting medium. Dilution with water is sometimes advisable from an optical point of view, on account of the increased visibility that it gives to many structures by lowering the index of refraction of the glycerin. But from the point of view of efficacious preservation it is always advisable to use undiluted glycerin, the strongest that can be procured.

Long soaking of tissues in glycerin of gradually increased strength is a necessary preliminary to mounting in all cases in which it is desired to obtain the best possible preparations, and to ensure that they shall keep well. If this soaking is done on the slide (the cover being removed and the object treated with fresh glycerin every one or two days), it is well to take the precaution recommended by Beale, of luting the edges of the cover so as to make the preparation air-tight, as glycerin is so highly hygroscopic that a drop of it exposed to the air rapidly diminishes in strength to a very considerable degree. In order to facilitate the removal of the cover in this process, the slide may be gently warmed by passing it two or three times through the flame of a spirit lamp. No preparation can be considered to be made *secundum artem* until every part of the object has been thoroughly impregnated with strong pure glycerin.

The shrinking that frequently occurs when delicate structures are brought into glycerin may generally be cured by this treatment; cells which first appear hopelessly collapsed gradually swell out to their normal forms and dimensions.

For closing glycerin mounts, the edges of the cover should first (after having been cleansed as far as possible from superfluous glycerin) be painted with a layer of *glycerin jelly;* as soon as this is set a coat of any of the usual cements may be applied. This has of course been for the last twenty years one of the commonplaces of histological technic, but that has not prevented somebody from recently describing the process at great length as new.

Glycerin dissolves carbonate of lime, and is therefore to be rejected in the preparation of calcareous structures that it is wished to preserve.

423. Extra-refractive Glycerin.—The already high index of refraction of glycerin (Price's glycerin, $n = 1{\cdot}46$) may be raised to about that of crown glass by dissolving suitable substances in the glycerin. Thus the refractive index of a solution of chloride of cadmium ($CdCl_2$)* in glycerin may be 1·504; that of a saturated solution of sulphocarbolate of zinc in glycerin may be 1·501; that of a saturated solution of Schering's † chloral hydrate (in crusts) in glycerin is 1·510; that of iodate of zinc in glycerin may be brought up to 1·56.‡ The clearing action of glycerin may thus be greatly increased, and the full aperture of homogeneous objectives brought to bear on objects mounted in one of the above-named solutions.

The sulphocarbolate of zinc solution§ may be prepared by taking equal parts by weight of Price's glycerin and sulphocarbolate of zinc crystals, mingling the two, and applying sufficient heat to boil the glycerin. The solution can be made in about an hour, but no fear need be had about boiling too long, as the longer this is done the less liability will there be for the solution to deposit crystals on the bottom of the bottle when cooled, which it will do if the temperature is only kept up long enough to dissolve the crystals. Filter while hot. The index may be brought up to 1·525 if desired by evaporating the solution somewhat, or by adding more carbolate.

424. BARFF'S **Boroglyceride** (see *Journ. Roy. Mic. Soc.*, 1882, p. 124).—This preparation may be obtained (price 1*s.* per bottle) from *The Kreochyle Company, Viaduct House, Farringdon Street, E.C.*, or all wholesale chemists.

425. Glycerin and Alcohol Mixtures.—These most useful fluids afford one of the best means of bringing delicate objects gradually from weak into strong glycerin. The object is mounted in a drop of the liquid, and left for a few hours or days, the mount not being closed. By the evaporation of the alcohol the liquid gradually increases in density, and after some time the mount may be closed, or the object brought into pure glycerin or glycerin jelly.

1. CALBERLA'S LIQUID:

Glycerin	1 part.
Alcohol	1 ,,
Water	1 ,,

A most valuable examination fluid. As already pointed out (p. 5), this liquid is in many cases to be preferred to alcohol for keeping fixed objects in until required for dissection or other further preparation.

2. I strongly recommend the following for very delicate objects:

---

* *Journ. Roy. Mic. Soc.*, ii, 1879, p. 346.

† *Ibid.* (N.S.), i, 1881, p. 943.

‡ *Ibid.*, p. 366.

§ *Ibid.*, iii, 1880, p. 1051.

Glycerin	1 part.
Alcohol	1 ,,
Water	2 parts.

3. Hæntsch's Liquid:

Glycerin	1 part.
Alcohol	3 parts.
Water	2 ,,

4. Jäger's Liquid (quoted from Vogt and Yung's *Traité d'Anat. comp. prat.*, p. 16):

Glycerin	1 part.
Alcohol	1 ,,
Sea water	10 parts.

426. Deane's **Glycerin Jelly** (from Frey's *Le Microscope*, p. 231).—120 grammes glycerin, 60 grammes water, 30 grammes gelatin. Dissolve the gelatin in the water, and add the glycerin. This, and the following glycerin jellies, must of course be used warm.

427. Lawrence's **Glycerin Jelly** (Davies, *Preparation and Mounting of Microscopic Objects*, p. 84).—"He takes a quantity of Nelson's gelatin, soaks it for two or three hours in cold water, pours off the superfluous water, and heats the soaked gelatin until melted. To each fluid ounce of the gelatin, *whilst it is fluid but cool*, he adds a fluid drachm of the white of an egg. He then boils this until the albumen coagulates and the gelatin is quite clear, when it is to be filtered through fine flannel, and to each ounce of the clarified solution add 6 drachms of a mixture composed of 1 part of glycerin to 2 parts of camphor water."

428. Beale's **Glycerin Jelly** (*How to Work*, &c., p. 57).—Gelatin or isinglass, soaked, melted, and clarified if desired, as in the last formula. To the clear solution add an equal bulk of strong glycerin.

429. Brandt's **Glycerin Jelly** (*Zeit. f. wiss. Mik.*, ii, 1880, p. 69; *Journ. Roy. Mic. Soc.*, iii, 1880, p. 502).—Melted gelatin 1 part, glycerin 1½ parts.

The gelatin to be soaked in water and melted in the usual way. After incorporating the glycerin, the mixture is to be filtered. This is a point of vital importance, as the gelatin of commerce is always mixed with particles of dust and minute threads. Swedish filtering-paper does not allow the fluid to pass through sufficiently, and flannel produces more threads than before. The following simple apparatus is found effective. A wide-necked bottle is broken in two, and the upper part taken. The neck is stopped with a cork having two holes bored in it. In the first hole a glass tube, about 20 cm. long, is inserted so as to project a little into the inside of the bottle, and on the outside it is bent sharply to one side and drawn out into a point of about 1½ to 2 mm. diameter. In the second hole a funnel-shaped filter is inserted so that the conical part is inside the bottle and the tube projects a few centimetres beyond the cork and the neck of the bottle. The apparatus is then placed so that the wide opening of the bottle and of the funnel is uppermost, and some spun glass is pressed into the lower conical part of the filter. In using the apparatus the funnel is filled with glycerin gelatin, and the bottle with hot water, which runs off slowly through the tube in the first hole and is constantly replenished.

Some drops of carbolic acid should be added to the fluid product of the filtering. For mounting, use warm by melting a small portion on the slide, the object having been previously soaked for some time in a small bottle of the medium warmed with a suitable apparatus.

430. Kaiser's **Glycerin Jelly** (*Bot. Cent.*, i, 1880, p. 25; *Journ. Roy. Mic. Soc.*, iii, 1880, p. 504).—One part by weight finest French gelatin is left for two hours in 6 parts by weight distilled water, 7 parts of glycerin are added, and for every 100 grammes of the mixture 1 gramme of concentrated carbolic acid. Warm for ten to fifteen minutes, stirring all the while, until the whole of the flakes produced by the carbolic acid have disappeared. Filter whilst warm through the finest spun glass laid wet in the filter. Use for mounting as above.

I prepared some of this jelly many years ago, and find it is still perfectly clear.

431. Seaman's **Glycerin Jelly** (*Amer. Mon. Mic. Journ.*, ii, 1881, p. 45; *Journ. Roy. Mic. Soc.* [N.S.], i, 1881, p. 534).—Dissolve isinglass in water, so that it makes a stiff jelly when at the ordinary temperature of the room, add one tenth as much glycerin, and a little solution of borax, carbolic acid, or camphor water. Filter whilst warm through muslin, and add a little alcohol.

432. Fol's **Glycerin Jellies** (*Lehrb.*, p. 138).

1. Melt together one volume of Beale's jelly (§ 428) and one half to one volume of water, and add 2 to 5 per cent. of salicylic acid solution, or carbolic acid or camphor.

2.

Gelatin	30	parts.
Water	70	„
Glycerin	100	„
Alcoholic solution of camphor	5	„

Prepare as before, adding the camphor last.

3.

Gelatin	20	parts.
Water	150	„
Glycerin	100	„
Alcoholic solution of camphor	15	„

433. Squire's **Glycerin Jelly** (Squire's *Methods and Formulæ*, &c., p. 84).—Soak 100 grms. of French gelatin in chloroform water, drain when soft, and dissolve with heat in 750 grms. of glycerin. Add 400 grms. of chloroform water with which has been incorporated about 50 grms. of fresh egg-albumen; mix thoroughly, and heat to boiling-point for about five minutes. Make up the total weight to 1550 grms. with chloroform water. Filter in a warm chamber.

434. Gilson's **Chloral Hydrate Jelly** (kindly communicated by Prof. Gilson).—1 vol. of gelatin, melted *secundum artem*, and 1 vol. of Price's glycerin. Mix, and add 1 vol. of chloral hydrate (*i. e.* add crystals of chloral until the volume of the mixture has increased by one half); warm till dissolved. This gives a very highly refractive aqueous mounting medium, which is found useful for opaque tissues that it is desired not to dehydrate.

A similar medium is published by Geoffroy, *Journ. de Botan.*, 1893, p. 55 (see *Zeit. f. wiss. Mik.*, ix, 4, 1893, p. 476). He dissolves, by the aid of as little heat as possible, 3 to 4 grms. of gelatine in 100 c.c. of 10 per cent. aqueous solution of chloral hydrate.

435. Stephenson's **Biniodide of Mercury and Iodide of Potas-**

sium (*Journ. Roy. Mic. Soc.* N.S., ii, 1882, p. 167).—A solution of the two salts in water. "This is very easily prepared by adding the two salts to the water until each shall be in excess; when this point of saturation has been reached the liquid will be found to have a refractive index of 1·68, by far the highest aqueous solution known to me. Its advantages from an optical point of view are considerable, and it may be used of any strength; commencing with pure water, with a refractive index of 1·33, we can go on progressively to 1·465, which represents glycerin, still on to 1·54 (Canada balsam), again onwards to 1·624, which represents bisulphide of carbon, to 1·658, which represents the monobromide of naphthalin, to 1·662, the equivalent of a solution of sulphur in bisulphide of carbon, until, undiluted, it finally reaches its own maximum of 1·680; thus we have the representatives of all these media and an infinite number of others in this one fluid."

This fluid is very dense, its specific gravity being 3·02. It is highly antiseptic.

"Its refractive index being 1·68, the visibility of diatoms, when mounted in it, is represented by the number 25 as compared with 11 in Canada balsam, in other words, the image is nearly two and a half times as strong. . . . . . . For muscular fibre, on the other hand, a strong solution is not suitable, since the high refractive power of the object approaches that of the medium, but as every other medium, of a lower index than 1·68 can, by dilution, be represented by it, any degree of visibility down to that of water can be obtained.

"For marine animals a weak solution is probably well adapted, as about a 1 per cent. solution (5 minims to the ounce) will give the specific gravity of sea water, with no appreciable difference in the refractive index."

Covers should be sealed with white wax, and the mounts finished with two or three coatings of gold-size and one of shellac.

I have experimented both with strong and with weak solutions. They are not adapted, I find, for the purposes of a permanent mounting medium. Tissues are well preserved, but the preparations are ruined by a precipitate which forms in the fluid. But as a temporary examination medium I have occasionally found this solution valuable. Its optical properties are wonderful; it allows of the examination of watery tissues, *without any dehydra-*

tion, in a medium of refractive index surpassing that of any known resinous medium.

436. **Monobromide of Naphthalin.** — See *Journ. Roy. Mic. Soc.*, 1880, p. 1043 (Abbe and van Heurck), and *Zool. Anz.*, 1882, p. 555 (Max Flesch).

437. Thompson's **High Refractive Medium.**—See *Journ. Roy. Mic. Soc.*, 1892, p. 902.

Resinous Media.

438. **Resins and Balsams.**—Resins and balsams consist of a vitreous or amorphous substance held in solution by an essential oil. By distillation or drying in the air they lose the essential oil and pass into the solid state. It is these solidified resins that should, in my opinion (and that, I believe, of the best microscopists), be employed for microscopical purposes; for the raw resins always contain a certain proportion of water, which makes it difficult to obtain a clear solution with the usual menstrua, is injurious to the optical properties of the medium and to its preservative qualities, and, further, especially hurtful to the preservation of stains. I therefore recommend that all solutions be made by heating gently the balsam or resin in a stove until it becomes brittle when cold, and then dissolving in an appropriate menstruum. Solid resins are now easily found in commerce.

Fol (*Lehrb.*, pp. 138–9) is of a different opinion.

Solutions made with volatile menstrua, such as xylol and chloroform, set rapidly, but become rapidly brittle. Solutions made with non-volatile media, such as turpentine, set much less rapidly, and pass much less rapidly into the brittle state. The former should, therefore, be employed whenever it is desired to have a mount that sets hard rapidly; but the latter should be employed whenever it is above all desired to have a mount that will prove as durable as possible.

According to my experience, *there is no such thing as a faultless resinous mounting medium for histological purposes.* Solutions of gum Damar in xylol are very beautiful from the physical point of view, and frequently afford a better definition of delicate detail than Canada balsam does. But I am convinced that *no Damar solution is perfectly stable.* A review of some old Damar mounts has shown that the majority of them have developed granules that have deteriorated the preparations to a greater or less extent. (These granules are in the worst cases large enough to at once attract attention even with low powers; at other times they are so small that they can only be seen with the highest powers, and in this case may be mistaken for normal elements of cells.) Xylol balsam and benzol balsam mounts are in the same case, but to a less degree. Chloroform balsam keeps much better, so far as granules are concerned. But it becomes very brown with age, and has the defect that it is injurious to stains made with coal-tar colours. Seiler's alcohol-balsam keeps remarkably well, but it also will not preserve the coal-tar stains or hæmatein stains. I use it for carmine stains. For coal-tar stains I now generally use *turpentine colophonium*. It gives very good definition of delicate detail, and keeps perfectly. (Dr. PAUL MAYER, however, writes me that turpentine solutions are not at all good for hæmatein stains). Turpentine colophonium has a rather low index of refraction for objects that require much clearing. For these I very frequently use *oil of cedar* wood in preference to any resinous medium. It gives perfect definition of all elements of the right index of visibility, and it keeps perfectly. With time it thickens sufficiently to hold the cover in place; or, if desired, preparations may be luted with Bell's cement. After using an oil immersion objective on a fresh mount, it is always easy to change the cover by floating it up with a drop of the oil placed at the side.

Another reason for preferring turpentine-colophonium where possible is that it does not shrink in drying nearly so much as the media made with volatile solvents. Workers who use benzol balsam, for instance, generally mount sections with strips of paper interposed between the slide and the cover, in order to prevent the sections from being crushed by the cover as it is drawn down in the process of drying. With turpentine media this is not necessary.

Still another motive is that turpentine media preserve the *index of visibility* of the preparations much longer than do media made with volatile menstrua. Preparations made with these last become so transparent in course of time that much fine detail is often lost. (Such mounts may, however, be revivified without removing the cover by putting them for a day or two into a tube of benzol; the benzol penetrates the balsam, and brings it down to a lower refractive index.) The *visibility* of minute structures is proportional to the *difference* between the refractive indices of the object and of the medium in which it is mounted. The majority of the elements of soft tissues are of an index of refraction somewhat superior to that of Canada balsam. It follows that by lowering the index of the balsam, increased visibility is obtained, and the desideratum in any case is to find a medium just low enough to give good *visibility*, and yet not so low as to seriously cut down the N.A. of the objectives employed.

UNIVERSITY OF CALIFORNIA

439. Choice of a Mounting Medium.—For the foregoing reasons I recommend *turpentine-colophonium* for general work; whilst for cases in which a more highly refractive medium is desired, I would recommend *oil of cedar* for coal-tar stains, and SEILER's *alcohol-balsam* for carmine stains. For hæmatein stains perhaps *xylol-balsam,* though those of my hæmatein preparations of which I have full notes have kept very well in either cedar oil or turpentine-colophonium. Xylol-balsam is certainly a very fine medium. I have merely wished to point out that it is not perfectly safe on the score of the possible formation of granules. (P. MAYER, *in litt.*, is of the same opinion.)

440. Canada Balsam.—Prepare with the solid balsam as above described, § 438. The usual menstrua are xylol, benzol, chloroform, and turpentine. Dissolve the solid balsam in one of these to the required consistence. The turpentine solution is to be preferred only in cases where it is desired to have a medium that sets very slowly, or in view of the better preservation of certain stains. (The objection to turpentine as a solvent is that it does not always give a homogeneous solution with Canada balsam as it does with colophonium.) For most other purposes the xylol solution is the best. If time be an object, a benzol solution should be preferred, as it sets much quicker than the xylol solution.

HEYS states that if the chloroform solution be poured into long, thin, half-ounce phials, corked up, and set aside for at least a month, the medium will be clearer and set much quicker than if the balsam is mixed with the chloroform at the time it is required for use (*Trans. Mic. Soc.*, Jan., 1865, p. 19; BEALE, p. 51).

SAHLI (*Zeit. f. wiss. Mik.*, 1885, p. 5) recommends *oil of cedar* as a menstruum.

MARTINOTTI (*Zeit. f. wiss. Mik.*, iv, 2, 1887, p. 159) says that he has obtained some beautiful solutions with oil of spike ("essence d'aspic rectifiée," of Duroriez, Paris). Unfortunately, he says, this medium will not preserve safranin stains.

441. SEILER'S **Alcohol Balsam** (*Proc. Amer. Soc. Mic.*, 1881, pp. 60–2; *Journ. Roy. Mic. Soc.* [N.S.], ii, 1882, pp. 126–7).—"Take a clear sample of Canada balsam and evaporate it in a water- or sand-bath to dryness; *i.e.* until it becomes brittle and resinous when cold. Dissolve this while warm in warm absolute alcohol and filter through absorbent cotton."

The advantage of this medium is stated to be that objects may be mounted in it direct from absolute alcohol, without previous treatment with an essential oil or other clearing agent; Seiler considers that by this means "shrivelling is avoided, as well as *the solution of fat in the cells.*"

The process of mounting direct from alcohol is not very easy to carry out, and I cannot recommend it for general work. But used in the ordinary way, after clearing by an essence, only xylol or the like, Seiler's solution is for some purposes admirable.

As stated above, I find that it is *one of the most stable solutions known to me.* (My stock, made up fifteen years ago, is still perfectly limpid, and has not sensibly darkened in colour.) It works pleasantly enough (if care be taken not to breathe on it during the process of mounting, as this may easily cause cloudiness). The definition is very fine, and the preservation of the preparations almost invariably perfect; my oldest preparations only show a few granules of little importance. Of course it has serious limitations. It cannot be used with the soluble coal-tar colours, and I find that it does not always preserve hæmatein stains, so that its chief employment is for carmine stains.

442. Damar (Gum Damar, or Dammar, or d'Ammar).—The menstrua are the same as for balsam, and the solution should be prepared in the same way. The most beautiful of all these mounting media is the solution of damar in xylol. Heat is not necessary to make the solution.

Minute directions (which I think unnecessary) for preparing a working solution are given by MARTINOTTI in *Zeit. f. wiss. Mik.*, iv, 2, 1887, p. 156, and in *Malpighia*, ii, 1888, p. 270; cf. also *Journ. Roy. Mic. Soc.*, 1889, p. 163.

FLEMMING, PFITZNER, and a writer signing C. J. M., all employ a mixture of benzol and turpentine (see *Arch. mik. Anat.*, xix, 1881, p. 322; *Sci. Gossip*, 1882, p. 257; *Journ. Roy. Mic. Soc.* [N.S.], iii, 1883, p. 145; *Morphol. Jahrb.*, vi, 1880, p. 469; *Journ. Roy. Mic. Soc.* [N.S.], ii, 1882, p. 583).

MAX FLESCH notes hereon (*Zool. Jahresber. f.* 1880, p. 51) that at Würzburg the ordinary dammar varnish of artists is employed.

JAMES (*Engl. Mech.*, 1887, p. 184; *Journ. Roy. Mic. Soc.*, 1887, p. 1061) also gives some, I think, superfluous formulæ for damar solutions; and still another new method is given, op. cit., 1890, p. 680.

A formula for a damar and mastic medium is given by SQUIRE in his *Methods and Formulæ*, &c., p. 84.

See further details concerning these solutions in former editions.

I quite acknowledge the special beauty of definition obtained by means of damar solutions; but I am convinced that *not one of these solutions can be depended on for really permanent preservations.* Sooner or later, sometimes after a few weeks or days, or it may be only after months or years, the granules mentioned in § 438 will make their appearance.

443. Colophonium.—A solution of colophonium in turpentine was first recommended by Kleinenberg. I find it to be *most highly recommendable.*

This medium sets very slowly, so that ample time is afforded for arranging objects in it. Kleinenberg warns against the employment of absolute alcohol as a solvent; the preparations are beautiful at first, but soon become spoiled by the precipitation of crystals or of an amorphous substance.

The turpentine solution *keeps perfectly limpid,* gives *very good definition,* and is altogether so excellent a medium that

I am surprised that it is not more used. It should be recommended to beginners. And, as stated in § 438, I consider that for many purposes it is perhaps the best and *most reliable* medium known. To make the solution, I add small lumps of colophonium to a quantity of rectified oil of turpentine kept in a stove, and when a sufficiently thick solution has been obtained, filter twice, the filtering being done in the stove. About a fortnight is required for the whole process. The solution should not be too thick, as it thickens somewhat with age. The palest sorts of colophonium should of course be selected.

444. Venice Turpentine for Mounting (Vosseler, *Zeit. f. wiss. Mik.*, vi, 3, 1889, p. 292, *et seq.*).—Vosseler strongly recommends this medium as having considerable advantages over Canada balsam or damar. Commercial Venice turpentine is mixed in a tall cylinder glass with an equal volume of 96 per cent. alcohol, allowed to stand in a warm place for three or four weeks, and decanted. It is stated that preparations may be mounted in this medium without previous clearing with essential oils or the like. The index of refraction being lower than that of the above-named balsams, delicate details are more distinctly brought out. Stains keep well in the medium, and Vosseler states that he possesses preparations made fifteen years ago that are perfectly well preserved.

This medium is also recommended by Suchannek (*ibid.*, vii, 4, 1891, p. 463). He advises that it be prepared with equal parts of Venice turpentine and neutral absolute alcohol (obtained by treating commercial absolute alcohol with calcined cupric sulphate and quicklime). The mixture should be agitated frequently and kept in a tile stove for a day or two until clear and sufficiently inspissated.

445. Copal Varnish.—I have seen tissues very instructively mounted in this medium. I have tried it myself, and failed. "Berry's Hard Finish," which is an easily obtainable copal varnish, has been highly praised for mounting purposes (see *Journ. Roy. Mic. Soc.*, 1887, p. 1064).

446. Cedar Oil.—I most highly recommend this oil, both as a temporary examination medium, and as a mounting medium. See § 438.

447. Castor Oil.—This was recommended as a mounting medium for certain delicate tissues (sections of eyes of Cephalopods) by GRENACHER (*Abhandl. naturf. Ges. Halle-a.-S.*, Bd. xvi; *Zeit. f. wiss. Mik.*, 1885, p. 244). This was with the idea that its low refractive index $n = 1{\cdot}49$, whilst Canada balsam $n = 1{\cdot}54$) would give a useful augmentation of visibility for the more refractive elements of the tissues.

With the objects with which I have experimented I have not found this to be the case.

448. Photographic Negative Varnish (for mounting large sections without cover-glasses).—See WEIGERT, *Zeit. f. wiss. Mik.*, iv, 2, 1887, p. 209.

449. Styrax and Liquidambar.—See *Journ. Roy. Mic. Soc.*, 1883, p. 741; ib., 1884, pp. 318, 475, 655, and 827; and the places there quoted. Also *Bull. Soc. Belge de Mic.*, 1884, p. 178; and FOL, *Lehrb.*, p. 141. These are very highly refractive media, which is just what is not wanted in general in histology.

450. Sandarac (LAVDOWSKY, from *Ref. Handbook Med. Sci.*, Supp. p. 438).—Gum sandarac 30 grs., absolute alcohol 50 c.c. This may, if desired, be diluted with an equal volume of absolute alcohol, and used for clearing sections.

CHAPTER XXII.

CEMENTS AND VARNISHES.

451. Introduction.—Thanks to the efforts of the dilettanti to outshine one another with neatly gaudy "rings," microscopical literature contains a goodly show of receipts for cements and varnishes. I have collected such as appear likely to be useful, rejecting all that relates merely to ornament.

Two, or at most three, of the media given below will certainly be found sufficient for all useful purposes. For many years I have used only one cement (Bell's). I recommend this as a cement and varnish; gold size may be found useful for turning cells; and Miller's caoutchouc cement may be kept for occasions on which the utmost solidity is required.

Marine glue is necessary for making glass cells.

Carpenter lays great stress on the principle that the cements or varnishes used for fluid mounts should always be such as contain *no mixture of solid particles;* he has always found that those that do, although they might stand well for a few weeks or months, yet always become porous after a greater lapse of time, allowing the evaporation of the liquid and the admission of air. All fluid mounts *should be ringed with glycerin jelly before applying a cement; by this means all danger of running in is done away with.* See § 453 and 454.

The above passage stands as it stood, italicised as here, in the first and second editions. It was translated and amplified, in a special paragraph, in the *Traité des Méth. techniques.* I may therefore be excused from hunting up the name of the anatomist who recently published as new this old old method, or the pages of the journals which reproduced his paper without protest.

The reader who requires more information concerning microscopical cements and varnishes than can be given in this chapter may consult with advantage the papers of

Aubert, *The Microscope*, xi, 1891, p. 150, and *Journ. Roy. Mic. Soc.*, 1891, p. 692; Beck, *The Microscope*, xi, 1891, pp. 338, 368, and *Journ. Roy. Mic. Soc.*, 1892, p. 293; and the last edition of Behrens' *Tabellen zum Gebrauch bei mikroskopischen Arbeiten* (Bruhn, Braunschweig, 1892).

452. Comparative Tenacity of Cements (see Behrens, *Zeit. f. wiss. Mik.*, ii, 1885, p. 54, and Aubert, *Amer. Mon. Mic. Journ.*, 1885, p. 227; *Journ. Roy. Mic. Soc.*, 1886, p. 173).—Behrens gives the palm to amber varnish; Aubert places Miller's caoutchouc cement at the head of the list, Lovett's cement coming halfway down, and zinc white cement at the bottom, with less than one quarter the tenacity of the caoutchouc cement.

453. The Paper Cell Method.—According to my experience, the best way to make a fluid mount safe is the following:—By means of two punches I cut out rings of paper of about a millimetre in breadth, and of about a millimetre smaller in diameter than the cover-glass. *Moisten* the paper ring with mounting fluid, and centre it on the slide. Fill the cell thus formed with mounting fluid; arrange the object in it; put the cover on; fill the annular space between the paper and the margin of the cover with glycerin jelly (a turn-table may be useful for this operation); and as soon as the gelatin has set turn a ring of Bell's or other cement on it.

For greater safety, the gelatin may of course be treated with bichromate according to Marsh's plan, next §.

454. Gelatin Cement (Marsh's *Section-cutting*, 2nd ed., p. 104).—Take half an ounce of Nelson's opaque gelatin, soak well in water, melt in the usual way, stir in 3 drops of kreasote, and put away in a small bottle. It is used warm.

When the ring of gelatin has become quite set and dry, which will not take long, it may be painted over with a solution of bichromate of potash made by dissolving 10 grains of the salt in an ounce of water. This should be done in the daytime, as the action of daylight is necessary to enable the bichromate to render the gelatin insoluble in water. The cover may then be finished with Bell's cement.

This process is particularly adapted for glycerin mounts.

455. Bell's **Cement.**—Composition unknown. May be obtained from the opticians or from J. Bell and Co., chemists, 338, Oxford Street, London.

This varnish flows easily from the brush, and sets quickly. For glycerin or other fluid mounts, the cover should be ringed as above described with glycerin jelly before applying the varnish. This precaution is especially necessary with glycerin. This is the best varnish for fluid mounts known to me. It is soluble in ether or chloroform. It is not attacked by oil of cedar.

456. Miller's **Caoutchouc Cement.**—Composition unknown. May be obtained from the opticians. A very tenacious and, which is frequently an important point, a quickly drying cement. It may be diluted by a mixture of equal parts of chloroform and strong alcohol (see Rousselet, *Journ. Quek. Club*, v, ii, 1895, p. 8).

457. Clarke's **Spirit-proof Cement.**—Mr. Ch. Rousselet has highly recommended this to me. It may be procured from Mr. J. Bolton, 25, Balshall Heath Road, Birmingham.

458. Asphalt Varnish (*Bitume de Judée*).—Unquestionably one of the best of these media, either as a cement or a varnish *provided it be procured of good quality.* It can be procured from the opticians or from the oil-shops. Kitton (*Month. Mic. Journ.*, 1874, p. 34) recommends asphalt dissolved in benzol with the addition of a small quantity of gold size.

459. Brunswick Black.—Best obtained from the opticians. A receipt for preparing it is given in Beale, *How to Work*, &c., p. 49.

"If a little solution of india-rubber in mineral naphtha be added to it, there is no danger of the cement cracking when dry." Carpenter states that without this addition it is brittle when dry. Brunswick black is soluble in oil of turpentine. A most useful cement, works easily and dries quickly. It can be recommended for turning cells.

460. Brunswick Black and Gold Size (Eulenstein, Beale, *How to Work, &c.*, p. 49).—Equal parts of Brunswick black and gold size with a very little Canada balsam.

461. **Gold Size.**—Receipts for preparing it may be found in the *Micrographic Dict.* or in Cooley's *Cyclopædia;* but it is certainly best to obtain it from the opticians or oil-shops. It is soluble in oil of turpentine. A good cement, *when of good quality*, and very useful for turning cells.

462. **Marine Glue.**—Found in commerce. Carpenter says the best is that known as G K 4.

It is soluble in ether, naphtha, or solution of potash. Its use is for attaching glass cells to slides, and for all cases in which it is desired to cement glass to glass.

Receipts for preparing it may be found in Beale, p. 40, or in Cooley's *Cyclopædia.*

463. Harting's **Gutta-percha Cement** (see Beale's *How to Work, &c.*, p. 49).—Marine glue serves the same purpose, viz. that of attaching cells to slides.

464. **India-rubber and Lime French Cement.**—See Beale, p. 58.

REESE LIBRARY OF THE UNIVERSITY OF CALIFORNIA

465. **Knotting** (*Journ. Roy. Mic. Soc.*, 1882, p. 745).—"Patent knotting" from oil and colour stores, exposed to the air until it has become of the proper consistency;—for mending cells and for preventing running-in of the finishing varnish (*Northern Microscopist*, ii, 1882).

466. **Turpentine, Venice Turpentine** (Csokor, *Arch. mik. Anat.*, xxi, 1882, p. 353; Parker, *Amer. Mon. Mic. Journ.*, ii, 1881, pp. 229–30; *Journ. Roy. Mic. Soc.* [N.S.], ii, 1882, p. 724).—Venice turpentine (Terebinthina veneta) is the liquid resinous exudation of *Abies larix*. It is seldom met with in a pure state. The following are the directions for preparing and using it given by Parker:

Dissolve true Venice turpentine in enough alcohol, so that after solution it will pass readily through a filter, and, after filtering, place in an evaporating dish, and by means of a sand-bath evaporate down to about three quarters of the quantity originally used. (The best way to tell when the evaporation has gone far enough is to drop some of the melted turpentine, after it has evaporated down to about three quarters of its original volume, into cold water; if on being taken out of the water it is hard and breaks with a vitreous fracture on being struck with the point of a knife, cease evaporation and allow to cool.)

Or (CSOKOR), common resinous turpentine of commerce is put in small pieces to melt over a water-bath, then poured into a suitable vessel and allowed to cool. It should form a brittle, dark brown mass, not yielding to the pressure of a finger. It is sometimes useful, in order to attain the right degree of hardness in the cold mass, to add a little resinous oil of turpentine to the melted mass, and then to evaporate for several hours over the water-bath.

This cement is used for closing glycerin mounts; it is applied in the following manner:—Square covers are used, and superfluous glycerin is cleaned away from the edges in the usual way.

The cement is then put on with a piece of wire bent at right angles (No. 10—12 wire is taken, and copper is the best, as it gives to the turpentine a greenish tinge); the short arm of the wire should be just the length of the side of the cover-glass. The wire is heated in a spirit lamp, plunged into the cement, some of which adheres to it, and then brought down flat upon the slide at the margin of the cover. The turpentine distributes itself evenly along the side of the cover, and hardens immediately, so that the slide may be cleaned as soon as the four sides are finished. It is claimed for this cement that it is perfectly secure, very handy, and never runs in. Parker saw this cement, or a similar one known as *Vendischer Damarlack*, exclusively used for glycerin mounts in the Pathological Laboratory at Vienna.

This is an extremely valuable method. It is very rapid and very safe. The cement sets hard in a few seconds.

467. Colophonium and Wax (KRÖNIG, *Arch. f. mik. Anat.*, 1886, p. 657; *Journ. Roy. Mic. Soc.*, 1887, p. 344).—Seven to nine parts of colophonium are added piecemeal to two parts of melted wax, the whole filtered and left to cool. For use, the mass is melted by placing the containing vessel in hot water. The cement is not attacked by water, glycerin, or caustic potash.

468. APÁTHY's **Cement for Glycerin Mounts** (*Zeit. f. wiss. Mik.*, vi, 2, 1889, p. 171).—Equal parts of hard (60° C. melting-point) paraffin and Canada balsam. Heat together in a porcelain capsule until the mass takes on a golden tint, and no longer emits vapours of turpentine. On cooling, this forms a hard mass, which is used by warming and applying with a glass rod or brass spatula. One application is enough. The cement does not run in, and never cracks.

469. JULIEN's **Balsam-Paraffin**, see *Journ. New York Mic. Soc.*, ix, 1893, p. 39, or *Journ. Roy. Mic. Soc.*, 1893, p. 567.

470. Paraffin.—*Temporary* mounts may be closed with pure paraffin, by applying it with a bent wire, as described, § 466.

471. Canada Balsam, or Damar.—Cells are sometimes made with these. They are elegant, but in my experience are not reliable for permanent mounts.

472. Amber Varnish.—As above mentioned, BEHRENS finds this cement to possess an extreme tenacity. He does not give the composition of his varnish, which was procured from E. Pfannenschmidt at Dantzic. The following is from COOLEY's *Cyclopædia*, art. "Varnish:"

"Take of amber (clear and pale) 6 lbs., fuse it; add of hot clarified linseed oil 2 gallons, boil it until it "strings" well, then let it cool a little and add of oil of turpentine 4 gallons or q. s."

Other receipts, l. c.

473. Amber and Copal Varnish (HEYDENREICH, *Zeit. f. wiss. Mik.*, 1885, p. 338).—An extremely complicated mode of preparation. The varnish may be obtained from Ludwig Marx, at 110, Moskowskaja Sastawa, St. Petersburg; or 79, Gaden, Vienna; or 1, Römerthal, Mayence.

474. Shellac Varnish (BEALE, p. 28).—Shellac should be broken into small pieces, placed in a bottle with spirit of wine, and frequently shaken until a thick solution is obtained. The *Micro. Dictionary* says that the addition of 20 drops of castor oil to the ounce is an improvement.

Untrustworthy, but useful for protecting balsam mounts from the action of oil of cedar.

For a method of preparing chemically pure shellac (a somewhat important matter), see Witt, *Zeit. f. wiss. Mik.*, 1886, p. 199.

For Seaman's shellac cement for attaching metal to glass, see *Journ. Roy. Mic. Soc.*, 1888, p. 520.

475. Sealing-Wax Varnish (*Micro. Dict.*, "Cements").—Add enough spirit of wine to cover coarsely powdered sealing-wax, and digest at a gentle heat. This should only be used as a varnish, never as a cement, as it is apt to become brittle and to lose its hold upon glass after a time.

476. Tolu Balsam Cement (Carnoy's *Biol. Cell.*, p. 129).

Tolu balsam	2 parts.
Canada balsam	1 part.
Saturated solution of shellac in chloroform	2 parts.

Add enough chloroform to bring the mixture to a syrupy consistence. Carnoy finds this cement superior to all others.

477. Stieda's **Zinc White or Red Cement** (*Arch. f. mik. Anat.*, 1866, p. 435).—Rub up oxide of zinc with turpentine, and add, stirring continually, for every drachm of the zinc oxide 1 ounce of a solution of damar in turpentine (of the consistency of thick syrup). This gives a *white* cement like Ziegler's. For a red cement take, instead of zinc, *cinnabar*, and take 2 drms. of the metal for each ounce of the damar solution. If the cement has become too thick with age, dilute with turpentine, ether, or chloroform. Recent authors who have discussed this cement are agreed that it is quite untrustworthy as a cement, though it may be useful as a finish.

478. Ziegler's **White Cement.**—Composition unknown. Is very much used on the Continent.

479. Kitton's **White Lead Cement** (*Month. Mic. Journ.*, 1876, p. 221).—Equal parts of white-lead, red-lead, and litharge (all in powder), ground together with a little turpentine until thoroughly incorporated, then mixed with gold size. The mixture should be thin enough to work with a brush. No more of the cement should be made than is required for present use, as it soon sets and becomes unworkable; but a stock of the materials may be kept ready ground in a bottle.

480. Lovett's **Cement** (*Journ. Roy. Mic. Soc.*, 1883, p. 786).—Two parts white-lead, two parts red oxide of lead (minium), three parts litharge. To be ground very fine, mixed dry, and kept so in a bottle. When required for use mix a little of the powder with gold size to the consistency of paint, taking care that no grit gets into it.

481. Aspinall's **Enamel.**—Stanley Kent (*Journ. Roy. Mic. Soc.*, 1890, p. 821) finds this of great use, both for ringing slides and making cells.

PART II.

SPECIAL METHODS AND EXAMPLES.

CHAPTER XXIII.

INJECTIONS—GELATIN MASSES.

482. Introduction.—Injection masses are composed of a coloured substance, technically termed the *colouring mass*, and of a substance with which that is combined, technically termed the *vehicle.*

The following formulæ are grouped mainly according to the nature of the vehicle.

For injections made for the study of the angiology of vertebrates, the student will do well to follow the masterly practice of Robin and Ranvier, consulting also, if necessary, the excellent instructions given in the *Lehrbuch der vergleichenden mikroskopischen Anatomie* of Fol. For injections of Invertebrates (and, indeed, for Vertebrates if it is desired to demonstrate the minute structure of environing tissues at the same time as the distribution of vessels) glycerin masses are frequently preferable to the gelatin masses chiefly employed by these authors; and I would recommend as particularly convenient the Prussian blue glycerin masses of Beale. Glycerin masses have the great advantage that they are used *cold.*

483. Nitrite of Amyl as a Vaso-dilator.—As stated above, glycerin masses are certainly very convenient, and give very good results from the scientific—not from the æsthetic—point of view. They have a great defect for the injection of fresh specimens—that is, those in which rigor mortis has not set in: they stimulate the contraction of arteries. In these cases it may be advisable to use nitrite of amyl as a vaso-dilator. The animal may be anæsthetised with a mixture of ether and nitrite of amyl, and finally killed with pure nitrite. Or, after killing by nitrite, a little nitrite of amyl in salt solution may be injected before the injection mass is thrown in. In any case it is advisable to add a little nitrite to the mass just before using. The relaxing power is very great (see Oviatt and Sargent, in *St. Louis Med. Journ.*, 1886, p. 207; and *Journ. Roy. Mic. Soc.*, 1887, p. 341).

Robin's *Masses.*

484. Robin's **Gelatin Vehicle** (*Traité*, p. 30).—Take some gelatin, of the sort known as "colle de Paris." (This gelatin is found in commerce in the form of thin sheets, marked with lozenge-shaped impressions of the cords which supported them whilst drying.) Soak it in cold water, then heat in water over a water-bath. One part of gelatin should be taken for every 7, 8, 9, or even 10 parts of water; it is a common error to employ solutions containing too much gelatin. The solution is now to be combined with one of the colouring masses given below.

This vehicle, like all gelatin masses, is liable to be attacked by mould if kept long; camphor and carbolic acid do not suffice to preserve it.

Chloral hydrate added to the mass will preserve it (Hoyer). A sufficient dose, at least 2 per cent., should be employed (see below, §§ 493, 494).

485. Robin's **Glycerin-gelatin Vehicle** (*Traité*, p. 32).—Dissolve in a water-bath 50 grms. of French gelatin ("colle de Paris") in 300 grms. of water, in which has been dissolved some arsenious acid; add of glycerin 150 grms., and of carbolic acid a few drops. Unlike the pure gelatin vehicles, this mass does keep indefinitely.

The colouring masses recommended for combination with the vehicles above described are made as follows:

486. Carmine Colouring Mass (*Traité*, p. 33).—Rub up in a mortar 3 grms. of carmine with a little water and enough ammonia to dissolve the carmine. Add 50 grms. of glycerin, and filter.

Prepare 50 grms. of acid glycerin (containing 5 grms. of acetic acid for every 50 grms. of glycerin), and add it by degrees to the carmine-glycerin, until a slightly acid reaction is obtained (as tested by very sensitive blue test-paper, moistened and held over the mixture).

One part of this mixture is to be added to 3 or 4 parts of the gelatin injection vehicle (*ante*, Formula 484), or of the glycerin-gelatin (§ 485).

487. Ferrocyanide of Copper Colouring Mass (*ibid.*, p. 34). Take—

(1) Ferrocyanide of potassium (concentrated solution)	20 c.c.
Glycerin	50 ,,
(2) Sulphate of copper (concentrated solution)	35 ,,
Glycerin	50 ,,

Mix (1) and (2) slowly, with agitation; at the moment of injecting combine with 3 volumes of vehicle.

488. Blue Colouring Mass (Prussian Blue) (*Robin's modification of Beale's formula, ibid.*, p. 35).

Take—

(A) Sulphocyanide of potassium (sol. sat.)	90 c.c.
Glycerin	50 ,,
(B) Liquid perchloride of iron at 30°	3 ,,
Glycerin	50 ,,

Mix slowly and combine the mixture with 3 parts of vehicle. It is well to add a few drops of HCl.

489. Cadmium Colouring Mass (*ibid.*, p. 36).

Take—

Sulphate of cadmium (sol. sat.)	40 c.c.
Glycerin	50 ,,
and	
Sulphide of sodium (sol. sat.)	30 ,,
Glycerin	50 ,,

Mix with agitation and combine with 3 vols. of vehicle.

490. Scheele's **Green Colouring Mass** (*ibid.*, p. 37).

Take—

Arseniate of potash (saturated solution)	80 c.c.
Glycerin	50 ,,
and	
Sulphate of copper (saturated solution)	40 ,,
Glycerin	50 ,,

Mix and combine with 3 vols. of vehicle.

Carmine-gelatin Masses.

491. Ranvier's **Carmine-Gelatin Mass** (*Traité technique*, p. 116).—Take 5 grms. Paris gelatin, soak it in water for half an hour, or until quite swollen and soft; wash it; drain it; put it into a test-tube and melt it, in the water it has absorbed, over a water-bath. When melted add slowly, and with continual agitation, a solution of carmine in ammonia, prepared as follows:—2½ grms. of carmine are rubbed up with a little water, and just enough ammonia, added drop by drop, to dissolve the carmine into a *transparent* solution.

When the carmine has been added to the gelatin, you will have about 15 c.c. of ammoniacal solution of carmine in gelatin, if the operations have been properly performed. This solution is to be kept warm on the water-bath, whilst you proceed to neutralise it by adding cautiously, drop by drop, with continual agitation, a solution of 1 part of glacial acetic acid in 2 parts of water. (When the mass is near neutrality, dilute the acetic acid still further.) The instant of saturation is determined by the smell of the solution, which gradually changes from ammoniacal to sour. As soon as the sour smell is perceived, the addition of acetic acid must cease, and the liquid be examined under the microscope. If it contains a granular precipitate of carmine, too much acid has been added, and the mass must be thrown away.

Ranvier states that by practice the operator learns to attain to perfect neutralisation almost infallibly in this way, and that this is the only way to attain to it. Trust must not be put in certain formulæ that profess to indicate the proportions of ammonia and acetic acid necessary for neutralisation, on account of the variation in strength of the solutions of ammonia kept in laboratories. The method proposed by Frey of determining beforehand the quantity of a known acetic solution that is necessary for neutralisation of a given quantity of the ammonia employed, is not infallible because it often happens that commercial gelatin is acid; in which case the proposed method would cause the operator to overpass the point of saturation.

The mass having been perfectly neutralised is strained through new flannel.

492. How to Neutralise a Carmine Mass (Ville, *Gaz. hebd.*

d. Sci. méd. de Montpellier, Fév., 1882 ; may be had separately from Delahaye et Lecrosnier, Paris).—Ville is of Ranvier's opinion that the method of titration recommended by Frey is defective, but for a different reason. When carmine is treated with ammonia a certain proportion of the ammonia combines with the carmine to form a transparent purple compound, and the rest of the ammonia remains in excess. It is this *excess* that it is required to neutralise precisely. In Frey's method a quantity of acid sufficient for the neutralisation of the *whole* of the ammonia employed is taken ; hence, naturally, the point of neutralisation is overstepped, and a granular mass is the result.

As to the acidity accidentally found in commercial gelatin, that source of error is easily eliminated. Instead of soaking the gelatin in water, it should be placed in a large funnel with a narrow neck, or better, in a stopcock funnel, and the whole should be placed under a tap, and a stream of water arranged in such a manner that the gelatin be constantly completely immersed. Washing for an hour or so in this way will remove all traces of acids mechanically retained in the gelatin.

As to the neutralisation of the colouring mass, Ville is of opinion that the criterion of neutrality given by Ranvier—the sour smell that takes the place of the ammoniacal odour—cannot be safely relied on in practice. He considers it greatly preferable to employ dichroic litmus paper (litmus paper sensitized so as to be capable of being used equally for the demonstration of acids and bases).

To prepare such a paper, the tincture obtained by decoction of cake litmus is slightly acidified by an excess of sulphuric acid. By this means the excess of alkali, or of alkaline carbonate, that is always present in litmus decoction, and which diminishes its sensibility as a reagent, is neutralised. The decoction is then heated and agitated with an excess of precipitated carbonate of baryta, and filtered.

The solution of litmus thus obtained is exposed to the air in wide vessels until its intense blue colour has given place to a reddish tint. Strips of white unsized paper are then dipped in it, and dried in the shade on stretched threads, in a place free from vapour of ammonia.

A shorter method consists in adding very dilute sulphuric acid, drop by drop, to the ordinary laboratory tincture of litmus, until the colour changes to red. Then, by adding successively traces of alkali and very dilute sulphuric acid, the reddish dichroic tint may be obtained, and the paper prepared with the solution as before.

The paper is used in the same way as ordinary litmus paper. A strip is moistened with distilled water and held as close as possible to the injection mass kept melted on a water-bath. It becomes blue at first, very rapidly and decidedly; but as fast as fresh quantities of acid are added this reaction becomes less evident, and at a certain moment the change of colour becomes very slow in making its appearance. It is then that the addition of acid should cease, and the operation is ended.

Very delicate sensitized paper may also be prepared with other reagents than litmus—for instance, with Nessler's reagent* or with alcoholic solution of hæmatoxylin, or with a solution made by adding a trace of dilute sulphuric acid to "liqueur orange No. 3" (a liquid found in commerce, and used for detecting acids); the solution takes on a gooseberry red colour.

The preparation of the injection mass is *facilitated* by employing acetic acid and ammonia of known strength. For the acetic acid it is sufficient to keep the glacial acid in a well-stoppered bottle. But this will not suffice for the ammonia, which is notably lowered in strength through the mere pouring from one bottle into another. Ville has imagined an apparatus which allows of withdrawing a known quantity without permitting any access of air to the stock solution. Description and figures, l. c.

With the exception of the processes above described, Ville prepares the injection mass exactly as Ranvier.

493. HOYER'S **Carmine-Gelatin Mass** (*Biol. Centralb.*, 1882, p. 21).—Take a concentrated gelatin solution and add to it a corresponding quantity of neutral carmine staining solution (l. c., p. 17). Digest in a water-bath until the dark violet-red colour begins to pass into a bright red tint. Then add 5—10 per cent. by volumes of glycerin, and at least 2 per cent. by weight of chloral, in a concentrated solution. After passing through flannel it can be kept in an open vessel under a bell-glass.

---

* Nessler's reagent may be prepared as follows:—Mercuric chloride, in powder, 35 grms.; iodide of potassium, 90 grms.; water, 1750 c.c. Heat gently till dissolved in a large basin; then add of stick caustic potash 320 grms., and 50 c.c. of saturated solution of mercuric chloride (WANKLYN). From COOLEY'S *Cyclopædia*, s. v. "Nessler's Test."

494. Fol's **Carmine-Gelatin Mass** (*Zeit. f. wiss. Zool.*, xxxviii, 1883, p. 492).

The following method of preparation has the advantage of producing masses that can be kept in the *dry state* for an indefinite length of time. (Fol finds that the addition of chloral hydrate to wet masses is not an efficient preservative.)

One kilog. of Simeon's photographic gelatin* is soaked for a couple of hours, until thoroughly soft, in a small quantity of water. The water is then poured off and the gelatin melted over a water-bath, and one litre of concentrated solution of carmine in ammonia is poured in with continual stirring. (The carmine solution is prepared by diluting strong solution of ammonia with three or four parts of water and adding carmine to saturation; the undissolved excess of carmine is removed by filtration just before the solution is added to the gelatin.)

To the mixture of gelatin and carmine, which should have a strong smell of ammonia, sufficient acetic acid is added to turn the dark purple colour of the mixture into the well-known blood-red hue. Exact neutralisation is not necessary. The mass is set aside until it has become firm, and is then cut up into pieces, which are tied up in a piece of tulle or fine netting. By means of energetic compression with the hand under water (it must be *acidulated* water, 0·1 per cent. acetic acid, otherwise the carmine will wash out: cf. *Journ. Roy. Mic. Soc.*, iv, part 3, 1884, p. 474) the mass is driven out through the meshes of the stuff in the shape of fine strings, which are washed for several hours in a sieve placed in running water in order to free them from any excess of acid or ammonia. The strings are then again melted, and the molten mass is poured on to large sheets of parchment paper soaked with paraffin, and the sheets are hung up to dry in an airy place. When dry the gelatin can easily be separated from the sheets, and may be cut into long strips with scissors and put away, protected from dust and damp, until wanted for use. In order to get the mass ready for use, all that is necessary is to soak the strips for a few minutes in water and melt them over a water-bath.

---

* This gelatin may be obtained either from the ordinary providers of articles used in photography, or direct from Simeon's Gelatin-fabrik, Winterthur, Switzerland. Two sorts, a hard and a soft, are sold; the softer is to be preferred on account of its lower point of fusion. Probably the photographic gelatins of Hinrichs, of Frankfurt, and of Coignet, of Paris, would answer equally well; as also the best English preparations.

The process may be simplified, without giving very greatly inferior results, as follows (*Lehrb.*, p. 13). Gelatin in sheets is macerated for two days in the above-described carmine solution, then rinsed and put for a few hours into water acidulated with acetic acid. It is then washed on a sieve for several hours in running water, dried on parchment paper, and preserved as above.

This injection mass is very well spoken of.

495. Other Carmine Gelatin Masses.—GERLACH'S **Carmine-Gelatin Mass** (see *Arch. f. mik. Anat.*, 1865, p. 148; and Ranvier's *Traité*, p 113). THIERSCH'S **Carmine-Gelatin Mass** (see *ibid.*). CARTER'S **Carmine-Gelatin Mass** (see BEALE, p. 113). DAVIES' **Carmine-Gelatin Mass** (see his *Prep. and Mounting of Mic. Objects*, p. 138).

Blue Gelatin Masses.

496. ROBIN'S **Prussian Blue Gelatin Mass** (see above, § 488).

497. RANVIER'S **Prussian Blue Gelatin Mass** (*Traité*, p. 119).—Twenty-five parts of a concentrated aqueous solution of soluble Prussian blue (prepared as directed below) mixed with 1 part of solid gelatin.

The mixture of the Prussian blue with the vehicle is effected in the following manner:

Weigh the gelatin, soak it in water for half an hour or an hour, wash it, and melt it in a test-tube, in the water it has absorbed, by heating over a water-bath. Put the solution of Prussian blue into another test-tube, and heat it on the same water-bath as the gelatin, so as to have the two at the same temperature. Pour the gelatin gradually into the Prussian blue solution, stirring continually with a glass rod. Continue stirring until the disappearance of the curdy precipitate that forms at first. (Some gelatins produce a *per-*

sistent precipitate; these must be rejected; but it must be borne in mind that the precipitate that invariably forms in even the best gelatins disappears if the heating be continued. It is essential to remember this when preparing Prussian blue and gelatin mass.) As soon as the glass rod has ceased to show blue granulations on its surface on being withdrawn from the liquid, it may be concluded that the Prussian blue is completely dissolved. Filter through new flannel, and keep the filtrate at 40° over a water-bath until injected.

The soluble Prussian blue for the above mass is prepared as follows:

498. Soluble Prussian Blue for Injection Masses (RANVIER, *ibid.*).—Make a concentrated solution of sulphate of peroxide of iron in distilled water, and pour it gradually into a concentrated solution of yellow prussiate of potash. There is produced a precipitate of insoluble Prussian blue. (An excess of prussiate of potash ought to remain in the liquid; in order to ascertain whether this is the case take a small quantity of the liquid and observe whether a drop of sulphate of iron still precipitates it.) Filter the liquid through a felt strainer, underneath which is arranged a paper filter in a glass funnel. The liquid at first runs clear and yellowish into the lower funnel; distilled water is then poured little by little on to the strainer; gradually the liquid issuing from the strainer acquires a blue tinge, which, however, is not visible in that which issues from the lower filter. Distilled water is continually added to the strainer for some days until the liquid begins to run off blue from the second filter. The Prussian blue has now become soluble. The strainer is turned inside out and agitated in distilled water; the Prussian blue will dissolve if the quantity of water be sufficient.

The solution may now be injected just as it is, or it may be kept in bottles till wanted, or the solution may be evaporated in a stove, and the solid residuum put away in bottle.

For injections, if a simple aqueous solution be taken, it should be *saturated*. Such a mass never transudes through the walls of vessels. Or it may be combined with one fourth of glycerin, or with the gelatin vehicle above described.

499. Soluble Prussian Blue (GUIGNET, see *Journ. de Microgr.*, 1889, p. 94; *Journ. Roy. Mic. Soc.*, 1889, p. 463; or previous editions of this work).

500. BRÜCKE'S **Soluble Berlin Blue** (*Arch. f. mik. Anat.*, 1865, p. 87).—Brücke first prepared it by taking a 10 per cent. solution of ferrocyanide of potassium, and precipitating by means of a dilute solution of sesquichloride of iron (taken in such a quantity as to contain just half as much chlorine as is necessary for the decomposition), and washing the precipitate on the filter until solubility is attained.

Later on he employed a greater excess of ferrocyanide, and took just so much dilute solution of chloride of iron that the weight of the dry chloride employed came to $\frac{1}{10}$ or $\frac{1}{8}$ of that of the ferrocyanide. The precipitate was washed on a filter (using the filtrate to wash with) until nothing but a clear yellow liquid filtered off, then washed with water until the water began to run off blue, then dried, pressed between blotting-paper in a press, the resulting mass broken in pieces and dried by exposure to the air.

A cheaper method is the following:

Make a solution of ferrocyanide of potassium containing 217 grammes of the salt to 1 litre of water.

Make a solution of 1 part commercial chloride of iron in 10 parts water.

Take equal volumes of each, and add to each of them twice its volume of a cold saturated solution of sulphate of soda. Pour the chloride solution into the ferrocyanide solution, stirring continually. Wash the precipitate on a filter until soluble, and treat as above described.

The concentrated solution of the colouring matter is to be gelatinised with just so much gelatin that the mass forms a jelly when cold.

501. THIERSCH'S **Prussian Blue Gelatin Mass** (*Arch. f. mik. Anat.*, i, 1865, p. 148).

Take—

(1) A solution of 1 part gelatin in 2 parts water.

(2) A saturated aqueous solution of sulphate of iron.

(3) A saturated aqueous solution of red prussiate of potash.

(4) A saturated aqueous solution of oxalic acid.

Now (A) mix 12 c.c. of the iron solution with one ounce of the gelatin solution at the temperature of 25° R.

Then (B) mix, at the same temperature, 24 c.c. of the prussiate solution with two ounces of the gelatin solution.

(C) To the latter mixture add first 24 c.c. of the oxalic acid solution, stir well, and then add the gelatin and iron mixture (A). Stir continually, keeping the temperature at from 20° to 25° R. until the whole of the Prussian blue is precipitated. Finally, heat over a water-bath to about 70° R. and filter through flannel.

502. Fol's **Berlin Blue Gelatin Mass** (*Zeit. f. wiss. Zool.*, xxxviii, 1883, p. 494).—A modification of Thiersch's formula, last §. 120 c.c. of a cold saturated solution of sulphate of iron are mixed with 300 c.c. of the warm gelatin solution. In a separate vessel 600 c.c. of the gelatin solution are mixed with 240 c.c. of a saturated solution of oxalic acid, and 240 c.c. of a cold saturated solution of red prussiate of potash are added to the mixture. The first mixture is now gradually poured into the second, with vigorous shaking, the whole is warmed for a quarter of an hour over a boiling water-bath, the mass is allowed to set, is pressed out into strings through tulle or netting, as described for the carmine mass, *supra*, § 494, and the strings are washed and spread out to dry on the prepared paper. (It is necessary to dry the strings without re-melting in this case, because the mass does not readily melt without the addition of oxalic acid.) In order to prepare the mass for injection, the strings are put to swell up in cold water, and then warmed with the addition of enough oxalic acid to allow of complete solution.

503. Hoyer's **Soluble Berlin Blue Gelatin Mass** (*Arch. f. mik. Anat.*, 1876, p. 649).—The filtered and not too much washed precipitate of soluble Berlin blue is brought in a little water on to a Graham's dialyser, and the external water changed until the solution begins to pass through the parchment. Dilute the solution and filter through filter-paper, an operation which becomes easy *after* dialysis. The solution may be injected pure (for lymphatics, for instance) or may be combined with gelatin. To do this, warm the solution almost to boiling-point, and add *gradually* a warm, thin solution of

gelatin until coagulation begins to set in. Strain through wetted flannel.

Gelatin Masses of other Colours.

504. Robin's **Cadmium Gelatin Mass** (see § 489).

505. Thiersch's **Lead Chromate Gelatin Mass** (*Arch. f. mik. Anat.*, 1865, p. 149).

Make—

(A) A solution of 1 part gelatin in 2 parts water.

(B) A solution of 1 part neutral chromate of potash in 11 parts water.

(C) A solution of 1 part nitrate of lead in 11 parts water.

Mix 4 parts of the gelatin solution with 2 parts of the lead solution, and in another vessel mix 4 parts gelatin solution with 1 part of the chromate solution. Heat both the mixtures to 25° R.; mix them together with continual stirring until all the chromate of lead is precipitated; heat over a water-bath to 70° R., and filter through flannel.

Fol remarks that this is the most beautiful of yellow masses, but *will not keep*, as the chromate of potash causes the gelatin to pass over gradually into the insoluble state.

506. Hoyer's **Lead Chromate Gelatin Mass** (*ibid.*, 1867, p. 136).

Take—

- One volume of a solution of gelatin containing 1 part of gelatin to 4 of water.
- One volume of cold saturated solution of bichromate of potash.
- And one volume cold saturated solution of sugar of lead (neutral plumbic acetate).

Filter the gelatin solution through flannel, and mix in the bichromate solution. Then *warm almost to boiling-point*, and add gradually the (warmed) sugar of lead solution. Allow the mass to cool down to body temperature, and inject at once. Another mode of preparation is as follows:—Mix the sugar of lead solution with part of the gelatin solution, mix the bichromate solution with the remaining gelatin solution, heat the latter mixture, and pour into it the former mixture (gradually), stirring continually.

If the solutions are mixed at a low temperature a lumpy granular precipitate is formed. Further, when solution of sugar of lead is added to a *hot* solution of bichromate of potash a rich orange-red precipitate is obtained; whilst if the solutions be mixed *cold* the precipitate is bright yellow.

If the solutions of the two salts be kept ready prepared, the injection mass may be mixed in less than a quarter of an hour. Its advantages are that, on account of the extremely fine state of division of the precipitate, the mass is almost transparent, and runs so freely that even lymphatics may be perfectly injected with it, whilst its intensity of colour makes the vessels much more distinct than the very pale mass of Thiersch (last Section). It is also easier to manage than Thiersch's mass, as it does not solidify so quickly. It shows well in the vessels by reflected, as well as by transmitted light.

507. FOL's **Lead Chromate Gelatin Mass** (*Lehrb.*, p. 15).

508. HOYER's **Silver Nitrate Yellow Gelatin Mass** (*Biol. Centralbl.*, ii, 1882, pp. 19, 22; *Journ. Roy. Mic. Soc.* [N.S.], iii, 1883, p. 142).—"A concentrated solution of gelatin is mixed with an equal volume of a 4 per cent. solution of nitrate of silver and warmed. To this is added a very small quantity of an aqueous solution of pyrogallic acid, which reduces the silver in a few seconds; chloral and glycerin are added as before" (see *ante*, HOYER's formula for carmine-gelatin, No. 493).

This mass is yellow in the capillaries and brown in the larger vessels. It does not change either in alcohol, chromic or acetic acid, or bichromate of potash, &c.

509. **Other Colours.**—HOYER's **Green Gelatin Masses** (*ibid.*).—Made by mixing a blue mass and a yellow mass. THIERSCH's **Green Gelatin Mass** (*Arch. f. mik. Anat.*, 1865, p. 149).—Made by mixing the blue mass, § 501, and the yellow mass, § 505. ROBIN's SCHEELE's **Green Gelatin Mass** (see § 490). HARTIG's **White Gelatin Mass** (see FREY, *Le Microscope*, p. 190). FREY's **White Gelatin Mass** (*ibid.*). TEICHMANN's **White Gelatin Mass** (*ibid.*, p. 191). FOL's **Brown Gelatin Mass** (*Zeit. f. wiss. Zool.*, xxxviii, 1883, p. 494). MILLER's **Purple Silver Nitrate Gelatin Mass** (see *Amer. Mon. Mic. Journ.*, 1888, p. 50; *Journ. Roy. Mic. Soc.*, 1888, p. 518; *Zeit. f. wiss. Mik.*, v, 3, 1888, p. 361). ROBIN's **Mahogany Gelatin Mass** (see § 487).

510. Ranvier's **Gelatin Mass for Impregnation** (*Traité*, p. 123).—Concentrated solution of gelatin, 2, 3, or 4 parts; 1 per cent. nitrate of silver solution, 1 part.

511. Fol's **Metagelatin Vehicle** (*Lehrb.*, p. 17).—The operation of injecting with the ordinary gelatin masses is greatly complicated by the necessity of injecting them warm. Fol proposes to employ metagelatin instead of gelatin.

If a slight proportion of ammonia be added to a solution of gelatin, and the solution be heated for several hours, the solution passes into the state of metagelatin, that is, a state in which it no longer coagulates on cooling. Colouring masses may be added to this vehicle, which may also be thinned by the addition of weak alcohol. After injection, the preparations are thrown into strong alcohol or chromic acid, which sets the mass.

CHAPTER XXIV.

INJECTIONS—OTHER MASSES (COLD).

512. Joseph's **White-of-Egg Injection Mass (Carmine)** (*Ber. naturw. Sect. Schles. Ges.* 1879, pp. 36—40; *Journ. Roy. Mic. Soc.* [N.S.], ii, 1882, p. 274).—"Filtered white of egg, diluted with 1 to 5 per cent. of carmine solution. . . . . This mass remains liquid when cold; it coagulates when immersed in dilute nitric acid, chromic or osmic acid, remains transparent, and is sufficiently indifferent to reagents."

For Invertebrates.

513. Bjeloussow's **Gum Arabic Mass** (*Arch. f. Anat. u. Phys.*, 1885, p. 379).—Make a syrupy solution of gum arabic and a saturated solution of borax in water. Mix the solutions in such proportions as to have in the mixture 1 part of borax to 2 of gum arabic. Rub up the transparent, almost insoluble mass with distilled water, added little by little, then force it through a fine-grained cloth. Repeat these operations until there is obtained a mass that is free from suspended gelatinous clots. (If the operation has been successful, the mass should coagulate in the presence of alcohol, undergoing at the same time a dilatation to twice its original volume.)

The vehicle thus prepared may be combined with any colouring mass except cadmium and cobalt.

After injection the preparation is thrown into alcohol, and the mass sets immediately, swelling up as above described, and consequently showing vessels largely distended.

Cold-blooded animals may be injected whilst alive with this mass. It does not flow out of cut vessels. Injections keep well in alcohol. Glycerin may be used for making them transparent.

If it be desired to remove the mass from any part of a preparation, this is easily done with dilute acetic acid, which dissolves it.

*Glycerin Masses.**

514. Beale's **Carmine Glycerin Mass** (*How to Work,* &c., p. 95).—Five grains of carmine are dissolved in a little water with the aid of about five drops of ammonia, and added to half an ounce of glycerin. Then add half an ounce of glycerin with eight or ten drops of acetic or hydrochloric acid, gradually, with agitation. Test with blue litmus paper, and if necessary add more acid till the reaction is decidedly acid. Then add half an ounce of glycerin, two drachms of alcohol, and six drachms of water. I have found this useful, but not so good as the Prussian blue injections.

515. Beale's **Prussian Blue Glycerin Mass** (*How to Work,* &c., p. 93).

Common glycerin . . .	1 ounce.
Spirits of wine	1 ,,
Ferrocyanide of potassium . .	12 grains.
Tincture of perchloride of iron .	1 drachm.
Water	4 ounces.

Dissolve the ferrocyanide in one ounce of the water and glycerin, and add the tincture of iron to another ounce. "These solutions should be mixed together *very gradually*, and well shaken in a bottle, *the iron being added to the solution of the ferrocyanide of potassium.* Next, the spirit and the water are to be added very gradually, the mixture being constantly shaken."

"*The water*" spoken of in the last sentence appears to mean the remaining three ouuces of water that were not mixed with the glycerin at first.

Injected specimens should be preserved in acidulated glycerin, otherwise the colour may fade.

516. Beale's **Acid Prussian Blue Glycerin Mass** (*ibid.*, p. 296).

Price's glycerin	2 fluid ounces.
Tinct. of sesquichloride of iron .	10 drops.
Ferrocyanide of potassium . .	3 grains.
Strong hydrochloric acid . .	3 drops.
Water	1 ounce.

---

* See the remarks on Glycerin Masses, § 483.

Proceed as directed above, dissolving the ferrocyanide in one half of the glycerin, the iron in the other, and adding the latter drop by drop to the former. Finally add the water and HCl. Two drachms of alcohol may be added to the whole if desired.

I consider this a most admirable formula. I possess some of this mass prepared many years ago, in which not the smallest flocculus has made its appearance. The Prussian blue appears to be in a state of true solution. The mass runs well, and has not so much tendency to exude from cut capillaries as might be supposed. Unfortunately it is a rather expensive preparation.

517. RANVIER'S **Prussian Blue Glycerin Mass** (*Traité*, p. 120).—Consists of the Prussian blue fluid, § 498, mixed with one fourth of glycerin.

518. Other Colours.—Any of the colouring masses, §§ 486 to 490, or other suitable colouring masses, combined with glycerin, either dilute or pure.

Aqueous Masses.

519. RANVIER'S **Prussian Blue Aqueous Mass** (*Traité*, p. 120).—The soluble Prussian blue, § 498, injected without any vehicle. It does not extravasate.

520. MÜLLER'S **Berlin Blue** (*Arch. f. mik. Anat.*, 1865, p. 150).—Precipitate a concentrated solution of Berlin blue by means of 90 per cent. alcohol.

The precipitate is very finely divided; the fluid is *perfectly neutral*, and much easier to prepare than the formula of Beale.

521. MAYER'S **Berlin Blue** (*Mitth. zool. Stat. Neapel*, 1888, p. 307).—A solution of 10 c.c. of tincture of perchloride of iron in 500 c.c. of water is added to a solution of 20 gr. of yellow prussiate of potash in 500 c.c. of water, allowed to stand for twelve hours, decanted, the deposit washed with distilled water on a filter until the washings come through dark blue (one to two days), and the blue dissolved in about a litre of water.

522. EMERY'S **Aqueous Carmine** (*ibid.*, 1881, p. 21).—To a 10 per

REESE LIBRARY OF THE UNIVERSITY

cent. ammoniacal solution of carmine is added acetic acid, with continual stirring, until the colour of the solution changes to blood-red through incipient precipitation of the carmine. The supernatant clear solution is poured off, and injected cold without further preparation. The injected organs are thrown at once into strong alcohol to fix the carmine. For injection of fishes.

523. LETELLIER'S **Vanadate of Ammonia and Tannin**.—See *Journ. Roy. Mic. Soc.*, 1889, p. 151, or previous editions.

524. TAGUCHI'S **Indian Ink** (*Arch. f. mik. Anat.*, 1888, p. 565; *Zeit. f. wiss. Mik.*, 1888, p. 503).—Chinese or (better) Japanese ink well rubbed up on a hone until a fluid is obtained that does not run when dropped on thin blotting-paper, nor form a grey ring round the drop. Inject until the preparation appears quite black, and throw it into some hardening liquid (not pure water).

I believe this will be found useful for many purposes, especially for work amongst Invertebrates, as well as for lymphatics, juice-canals, and the like.

Celloidin Masses.

525. SCHIEFFERDECKER'S **Celloidin Masses** (*Arch. Anat. u. Phys.*, 1882 [*Anat. Abth.*], p. 201). (For Corrosion preparations.) See previous editions of this work, or WHITMAN'S *Methods in Microscopical Anatomy.*

526. HOCHSTETTER'S **Modification of** SCHIEFFERDECKER'S **Mass** (*Anat. Anz.*, 1886, p. 51; *Journ. Roy. Mic. Soc.*, 1888, p. 159).

Other Masses.

527. BUDGE'S **Asphaltum Mass.**—See *Arch. f. mik. Anat.*, xiv, 1877, p. 70, or previous editions.

528. HOYER'S **Shellac Mass** (*Arch. f. mik. Anat.*, 1876, p. 645). See previous editions.

This method, with some slight modifications of detail, has been recommended by BELLARMINOW (*Anat. Anz.*, 1888, p. 650; see also *Zeit. f. wiss. Mik.*, v, 4, 1888, p. 523, and *Journ. Roy. Mic. Soc.*, 1889, p. 150).

529. HOYER'S **Oil-colour Masses** (*Internat. Monatschr. f. Anat.*, 1887, p. 341; see also *Zeit. f. wiss. Mik.*, 1888, p. 80, and *Journ. Roy. Mic. Soc.*, 1888, p. 848). PANSCH'S **Starch Mass** (see *Arch. f. Anat. u. Entw.*, 1877, p. 480; 1880, pp. 232, 371; 1881, p. 76; 1882, p. 60; 1883, p. 265; and a modification of the same by GAGE, *Amer. Mon. Mic. Journ.*, 1888, p. 195; and *Journ. Roy. Mic. Soc.*, 1888, p. 1056). **Teichmann's Linseed-Oil**

Masses (see *S. B. Math. Kl. Krakau Akad.*, vii, pp. 108, 158; *Journ. Roy. Mic. Soc.*, 1882, pp. 125 and 716, and 1895, p. 704).

530. Natural Injections (ROBIN, *Traite*, p. 6).—To preserve these throw the organs into a liquid composed of 10 parts of tincture of perchloride of iron and 100 parts of water.

RETTERER and ZELLNER use solution of Müller, see *Journ. Roy. Mic. Soc.*, 1894, p. 641.

CHAPTER XXV.

MACERATION AND DIGESTION.

Maceration.

531. Methods of Dissociation.—It is sometimes necessary, in order to obtain a complete knowledge of the forms of the elements of a tissue, that the elements be artificially separated from their place in the tissue and separately studied after they have been isolated both from neighbouring elements and from any interstitial cement-substances that may be present in the tissue. Simple teasing with needles is often insufficient to effect the desired isolation, as the cement-substances are often tougher than the elements themselves, so that the latter are torn and destroyed in the process. In this case recourse must be had to maceration processes, by which is here meant treatment with media which have the property of dissolving or at least softening the cement-substances or the elements of the tissue that it is not wished to study, whilst preserving the forms of those it is desired to isolate. When this softening has been effected the isolation is completed by teasing, or by agitation with liquid in a test-tube, or by the method of tapping, which last gives in many cases (many epithelia, for instance) admirable results which could not be attained in any other way. The macerated tissue is placed on a slide and covered with a thin glass cover supported at the corners on four little feet made of pellets of soft wax. By tapping the cover with a needle it is now gradually pressed down, whilst at the same time the cells of the tissue are segregated by the repeated shocks. When the segregation has proceeded far enough, mounting medium may be added, and the mount closed.

The student will do well not to neglect this simple method, which is one that it is most important to be acquainted with.

A good material for making *wax feet* is obtained (VOSSELER, *Zeit. f. wiss. Mik.*, vii, 4, 1891, p. 461) by melting white wax and stirring into it one half to two thirds of Venice turpentine. Care must be taken if the operation be performed over a naked flame, as the turpentine vapours are inflammable.

532. Iodised Serum.—The preparation of this reagent has been given in Chap. XXI. The manner of employing it for maceration is as follows :—A piece of tissue smaller than a pea must be taken, and placed in 4 or 5 c.c. of weakly iodised serum in a well-closed vessel. After one day's soaking the maceration is generally sufficient, and the preparation may be completed by teasing or pressing out, as indicated above; if not, the soaking must be continued, fresh iodine being added as often as the serum becomes pale by the absorption of the iodine by the tissues. By taking this precaution, the maceration may be prolonged for several weeks.

It is obvious that these methods are intended to be applied to the preparation of *fresh* tissues, the iodine playing the part of a fixing agent with regard to protoplasm, which it slightly hardens.

533. Artificial Iodised Serum (FREY, *Le Microscope,* p. 131; RANVIER, *Traité,* p. 77).

The formula has been given in Chap. XXI. Ranvier states that he has been unable to obtain good results, for purposes of maceration, by this method.

534. Alcohol.—RANVIER employs one-third alcohol (1 part of 36° alcohol to 2 parts of water). Epithelia will macerate well in this in twenty-four hours. Ranvier finds that this mixture macerates more rapidly than iodised serum.

Other strengths of alcohol may be used, either stronger (equal parts of alcohol and water) or weaker ($\frac{1}{4}$ alcohol, for isolation of the nerve-fibres of the retina, for instance—THIN).

All observers are agreed that one-third alcohol is a macerating medium of the highest order; LIST (*Zeit. f. wiss. Mik.,* 1885, p. 511) states that for glandular structures it should be used with precaution, on account of swellings that it produces in the cells, and that Müller's solution, or osmic acid, should be preferred for such objects.

535. Salt Solution.—10 per cent. solution of sodium chloride is a well-known and valuable macerating medium.

536. Moleschott and Piso Borme's **Sodium Chloride and Alcohol** (Moleschott's *Untersuchungen zur Naturlehre,* xi, pp. 99—107; Ranvier, *Traité,* p. 242).—10 per cent. solution of sodium chloride, 5 volumes; absolute alcohol, 1 volume.

For vibratile epithelium, Ranvier finds the mixture inferior to one-third alcohol.

537. Formaldehyde.—Gage has tested formaldehyde as a dissociating agent. He recommends the addition of two parts of formalin (40 per cent. solution of formaldehyde) to 1000 parts of normal salt solution. He states that the results are highly satisfactory, the mixture acting quickly and yet retarding deterioration for some time (quoted from Fish, *Proc. Am. Mic. Soc.,* xvii, 1895, p. 328).

538. Chloral Hydrate.—In not too strong solution, from 2 to 5 per cent., for instance, chloral hydrate is a mild macerating agent that admirably preserves delicate elements. Lavdowsky (*Arch. f. mik. Anat.,* 1876, p. 359) recommends it greatly for salivary glands. Hickson (*Quart. Journ. Mic. Sci.,* 1885, p. 244) recommends it for the study of the retina of Arthropods.

539. Caustic Potash, Caustic Soda.—These solutions must be employed *strong,* 35 to 50 per cent. (Moleschott); so employed they do not greatly alter the forms of cells, whilst weak solutions destroy all the elements. (Weak solutions may, however be employed for dissociating the cells of epidermis, hairs, and nails.) The strong solutions may be employed by simply treating the tissues with them on the slide. To make permanent preparations, the alkali should be neutralised by adding acetic acid, which forms with caustic potash acetate of potash, a well-known mounting medium (see Behrens, Kossel, and Schiefferdecker, *Das Mikroskop,* i, 1889, p. 156). It has been found by S. H. and S. P. Gage (*Proc. Amer. Soc. of Microscopists,* 1889, p. 35; *Zeit. f. wiss. Mik.,* vii, 3, 1890, p. 349) that instead of acetic acid, 60 per cent. acetate of potash solution, employed in considerable quantity and if desired with addition of 1 per cent. of acetic acid, may be used, the preparations either being mounted therein, or in glycerin or glycerin jelly. They may be stained if the acetate be first washed out by treatment for twenty-four hours with alum solution.

540. Sulphocyanides of Ammonium and Potassium (STIRLING, *Journ. Anat. and Phys.*, xvii, 1883, p. 208).—10 per cent. solution of either of these salts is, according to Stirling, an admirable dissociating medium for epithelium. Macerate small pieces for twenty-four to forty-eight hours.

If a crystalline lens be macerated as above its fibres become beaded or moniliform.

541. SOULIER'S **Sulphocyanide Mixtures** (*Travaux de l'Inst. zool. de Montpellier*, Nouv. Sér., 2, 1891, p. 171). Soulier has found that Stirling's solution greatly deteriorates cellular elements, but that good results are obtained by combining it with a fixing agent. He prepared the following series of mixtures:

Nos.	1.	2.	3.	4.	5.	6.	7.	8.
Sulphocyanide of ammonium or potassium of 10 per cent., 5 per cent., 2·5 per cent., or 1·25 per cent. strength	20 c.c.	30 c.c.	35 c.c.	36 c.c.	37 c.c.	38 c.c.	39 c.c.	39·5 c.c.
Solution of Ripart and Petit . . .	20 c.c.	10 c.c.	5 c.c.	4 c.c.	3 c.c.	2 c.c.	1 c.c.	0·5 c.c.

The best results were obtained with series made with a 2 per cent. solution of sulphocyanide.

Soulier also obtained good results by combining liquid of Ripart and Petit with artificial serum of Kronecker instead of sulphocyanide, or with pepsin, eau de Javelle, 10 per cent. sulphate of soda, or 1·5 per cent. solution of caustic soda.

And he further found that good results are obtained by combining solutions of chloride of sodium, or solutions of caustic potash or soda, with any of the usual fixing agents.

542. Saliva, Artificial (for embryology of nerve and muscle) (CALBERLA'S formulæ, *Arch. f. mik. Anat.*, xvi, 1879, p. 471, *et seq.*).—After having made trial of various different macerating agents, with the object of obtaining isolation of the developing muscle and nerve of embryos of *Amphibia* and

Ophidia, Calberla found that the best results were obtained by means of Czerny's mixture of saliva and solutio Mülleri. This led him to imagine an artificial saliva, which on trial gave results as good as those obtained by natural saliva, or even better.

Second formula (the first formula is suppressed, as being more complicated, and not giving better results):

Potassium chloride	0·4
Sodium chloride	0·3
Phosphate of soda	0·2
Calcium chloride	0·2
	1·1

This is dissolved in 100 parts of water, saturated with carbonic acid, and the solution combined with water and solutio Mülleri, one volume of the solution being combined with half a volume of Müller's solution and a volume of water.

In either case the Müller's solution may be replaced by a 2½ per cent. solution of chromate of ammonia. The best results were obtained when the solutions were saturated with the CO_2 just before using.

The tissues are isolated by teasing and shaking, and specimens mounted in concentrated acetate of potash.

543. LANDOIS'S **Solution** (*Arch. f. mik. Anat.*, 1885, p. 445).

Saturated sol. of neutral chromate of ammonia	5 parts.
Saturated sol. of phosphate of potash	5 „
Saturated sol. of sulphate of soda	5 „
Distilled water	100 „

To be used in the same way as chromic acid:—Small pieces of tissue are macerated for one to three, or even four or five days, in the liquid, then brought for twenty-four hours into ammonia carmine diluted with one volume of the macerating liquid.

GIERKE particularly recommends this liquid for all sorts of macerations, but especially for the central nervous system, for which he finds it superior to all other agents. It is also recommended for the same purpose by NANSEN (v. *Zeit. f. wiss. Mik.*, v, 2, 1888, p. 242).

544. Permanganate of Potash.—Has an action similar to that of osmic acid, but more energetic. Is recommended, either alone or combined with alum, as the best dissociating agent for the fibres of the cornea (Rollett, *Stricker's Handbuch*, p. 1108).

545. Chromic Acid.—Generally employed of a strength of about 0·02 per cent. Specially useful for nerve tissues and smooth muscle. Twenty-four hours' maceration will suffice for nerve-tissue. About 10 c.c. of the solution should be taken for a cube of 5 mm. of the tissue (Ranvier).

546. Bichromate of Potash.—0·2 per cent.

547. Müller's Solution.—Same strength.

548. Müller's Solution and Saliva (see above, § 542).

549. Brock's **Medium** (for nervous system of Mollusca, *Intern. Monatsch. f. Anat.*, i, 1884, p. 349).—Equal parts of 10 per cent. solution of bichromate of potash and visceral fluid of the animal.

550. Möbius's **Media** (quoted from *Zeit. f. wiss. Mik.*, iii, 3, 1886, p. 402).

1. One part of sea water with 4 to 6 parts of 0·5 per cent. solution of bichromate of potash.
2. 0·25 per cent. chromic acid, 0·1 per cent. osmic acid, 0·1 per cent. acetic acid, dissolved in sea water. For Lamellibranchiata. Macerate for several days.

551. Gage's **Picric Alcohol** (*Proc. Amer. Soc. of Microscopists*, 1890, p. 120; *Zeit. f. wiss. Mik.*, ix, 1, 1892, pp. 87, 88).—95 per cent. alcohol, 250 parts; water, 750; picric acid, 1. Recommended for most tissues, but especially for epithelia and smooth and striated muscle. This is also much recommended by Hopkins in the same place. A few hours' maceration is generally sufficient.

552. Osmic Acid.—0·1 per cent., for from a few minutes to a fortnight (cortex of cerebrum—Rindfleisch). May be followed by maceration in glycerin.

553. Osmic and Acetic Acid (the HERTWIGS' *Liquid, Das Nervensystem u. die Sinnesorgane der Medusen*, Leipzig, 1878, and *Jen. Zeitschr.*, xiii, 1879, p. 457; *Journ. Roy. Mic. Soc.*, iii, 1880, p. 441, and [N.S.] iii, 1883, p. 732).

0·05 per cent.	osmic acid	. . .	1 part.
0·2 ,,	acetic acid	. . .	1 ,,

Medusæ are to be treated with this mixture for two or three minutes, according to size, and then washed in repeated changes of 0·1 per cent. acetic acid until all traces of free osmic acid are removed; they then remain for a day in 0·1 per cent. acetic acid, are washed in water, stained in Beale's carmine (in order to prevent the osmium from over-blackening, and to assist the maceration), and are preserved in glycerin.

For *Actiniæ* the osmic acid is taken weaker, 0·04 per cent.; both the solutions are made with sea water; and the washing out is done with 0·2 per cent. acetic acid. If the maceration is complete, stain with picro-carmine; if not, with Beale's carmine.

554. BÉLA HALLER'S **Mixture** (*Morphol. Jahrb.*, xi, p. 321).—One part glacial acetic acid, 1 part glycerin, 2 parts water. Specially recommended for the central nervous system of Mollusca (Rhipidoglossa). A sufficient degree of maceration is obtained in thirty to forty minutes, the cells showing less shrinkage than with other liquids.

555. Nitric Acid.—Most useful for the maceration of muscle. The strength used is 20 per cent. After twenty-four hours' maceration in this, isolated muscle-fibres may generally be obtained by shaking the tissue with water in a test-tube. Preparations may afterwards be washed with water and put up in strong solution of alum, in which they may be preserved for a long time (HOPKINS, *Proc. Amer. Soc., of Microscopists*, 1890, p. 165; *Zeit. f. wiss. Mik.*, ix, 1, 1892, p. 86).

Maceration is greatly aided by heat, and at a temperature of 40° to 50° C. may be sufficiently complete in an hour (GAGE).

556. Nitric Acid and Chlorate of Potash (KÜHNE'S method, *Ueber die peripherischen Endorgane*, &c., 1862; RANVIER, *Traité*,

p. 79).—Chlorate of potash is mixed, in a watch-glass, with four times its volume of nitric acid. A piece of muscle is buried in the mixture for half an hour, and then agitated with water in a test-tube, by which means it entirely breaks up into isolated fibres.

557. Sulphuric Acid (Ranvier, *Traité*, p. 78).—Sulphuric acid has been employed by Max Schultz for isolating the fibres of the crystalline.

Macerate for twenty-four hours in 30 grms. of water, to which are added 4 to 5 drops of concentrated sulphuric acid. Agitate.

Odenius found very dilute sulphuric acid to be the best reagent for the study of nerve-endings in tactile hairs. He macerated hair-follicles for from eight to fourteen days in a solution of from 3 to 4 grains of "English sulphuric acid" to the ounce of water.

Hot concentrated sulphuric acid serves to dissociate horny epidermic structures (horn, hair, nails).

558. Oxalic Acid.—Maceration for many days in concentrated solution of oxalic acid has been found useful in the study of nerve-endings.

559. Schiefferdecker's **Methyl Mixture** (for the retina) (*Arch. f. mik. Anat.*, xxviii, 1886, p. 305).—Ten parts of glycerin, 1 part of methyl alcohol, and 20 parts of distilled water. Macerate for several days (perfectly fresh tissue).

560. **Lysol** (Reinke, *Anat. Anz.*, viii, 1892, p. 582; *Zeit. f. wiss. Mik.*, x, 2, 1893, p. 224).—Lysol is an industrial product consisting in a solution of cresol in a neutral soap, and is used as an antiseptic. According to Reinke, it possesses a very rapid macerating action. He uses a 10 per cent. solution in distilled water or in water containing alcohol and glycerin. Spermatozoa of the rat are said to be resolved into fibrils in a few minutes. The cortical cells of hairs are likewise resolved into fibrils in a few minutes. Epithelial cells of Salamandra are said to be dissociated instantaneously. Nuclear chromatin is destroyed, bringing into evidence the reticulated caryoplasm.

For some conclusions founded on this reaction, see *Arch. f. mik. Anat.*, xliii, 3, 1894, p. 398, *et seq.*

Digestion.

561. Beale's **Digestion Fluid** (*Archives of Medicine*, i, 1858, pp. 296—316).—The mucus expressed from the stomach glands of the pig is rapidly dried on glass plates, powdered, and kept in stoppered bottles. It retains its properties for years. Eight tenths of a grain will dissolve 100 grains of coagulated white of egg.

To prepare the digestion fluid, the powder is dissolved in distilled water, and the solution filtered. It filters readily. Or the powder may be dissolved in glycerin. The tissues to be digested may be kept for some hours in the liquid at a temperature of 100° F. (37° C.).

562. Brücke's **Digestion Fluid** (from Carnoy's *Biologie cellulaire*, p. 94).

Glycerinated extract of pig's stomach . .	1 vol.
0·2 per cent. solution of HCl . . .	3 vols.
Thymol, a few crystals.	

563. Bickfalvi's **Digestion Fluid** (*Centrabl. f. d. med. Wiss.*, 1883, p. 833).—One grm. of dried stomachal mucosa is mixed with 20 c.c. of 0·5 per cent. hydrochloric acid, and put into an incubator for three or four hours, then filtered. Macerate the tissue in the solution for not more than half an hour to an hour.

564. Kuskow's **Digestion Fluid** (*Arch. f. mik. Anat.*, xxx, p. 32; cf. *Zeit. f. wiss. Mik.*, iv, 3, 1887, p. 384).—One part of pepsin dissolved in 200 parts of 3 per cent. solution of oxalic acid. The solution should be freshly prepared, and the objects (sections of hardened Ligamentum Nuchæ) remain in it at the ordinary temperature for ten to forty minutes.

565. Schiefferdecker's **Pancreatin Digestion Fluid** (*Zeit. f. wiss. Mik.*, iii, 4, 1886, p. 483).—Solution of pancreatin in water. Schiefferdecker employs the "Pankreatinum siccum" prepared by Dr. Witte, Rostock. A saturated solution is made in distilled water, cold, and filtered. Pieces of tissue (epidermis) are macerated in it for three to four hours at about body temperature. Nuclei are preserved, and the forms of prickle-cells well shown.

566. Kühne's **Trypsin Methods** (see *Unters. a. d. Phys. Inst. Univ. Heidelberg*, i, 2, 1877, p. 219).—Very complicated.

567. Gedoelst's **Methods** (see *La Cellule*, iii, 1887, p. 117, and v, 1889, p. 126; also *Zeit. f. wiss. Mik.*, vii, 1, 1890, p. 57).

CHAPTER XXVI.

CORROSION, DECALCIFICATION, AND BLEACHING.

Corrosion.

568. Caustic Potash, Caustic Soda, Nitric Acid.—Boiling, or long soaking in a strong solution of either of these, is an efficient means of removing soft parts from skeletal structures (appendages of Arthropods, spicula of sponges, &c.).

569. Eau de Javelle (Hypochlorite of Potash) (NOLL'S METHOD, *Zool. Anzeig.*, 122, 1882, p. 528).—Noll remarks that the usual method of preparing the skeleton of siliceous sponges and similar structures by corroding away the soft parts by means of caustic potash has many disadvantages, of which a principal one is that the spicula are not preserved in their normal positions. He therefore proceeds as follows :—A piece of sponge is brought on to a slide and treated with a few drops of eau de Javelle, in which it remains until all soft parts are dissolved. (With thin pieces this happens in twenty to thirty minutes.) The preparation is then cautiously treated with acetic acid, which removes all precipitates that may have formed, and treated with successive alcohols and oil of cloves, and finally mounted in balsam.

The same process is stated to be applicable to calcareous structures. I feel convinced, however, that if the structures are *delicate,* they will suffer, or be entirely destroyed.

570. Eau de Labarraque (Hypochlorite of Soda) may be used in the same way as eau de Javelle. Looss (*Zool. Anz.*, 1885, p. 333) finds that either of these solutions will completely dissolve chitin in a short time with the aid of heat. For this purpose the commercial solution should be taken concentrated and boiling. A formula for making it is given in § 597.

If solutions diluted with 4 to 6 volumes of water be taken, and chitinous structures be macerated in them for twenty-four hours or more, according to size, the chitin is not dissolved, but becomes transparent, soft, and permeable to staining fluids, aqueous as well as alcoholic. The most delicate structures, such as nerve-endings, are stated not to be injured by the treatment. The method is applicable to Nematodes and their ova, an object well known for the resistance they oppose to ordinary reagents, and also to the removal of the albumen from ova of Amphibia, &c.

This is undoubtedly a valuable method.

571. ALTMANN's **Corrosion Method** (*Arch. f. mik. Anat.*, 1879, p. 471).—Whilst almost all animal tissues are very quickly destroyed by eau de Javelle, yet fats, and particularly fats hardened by osmic acid, withstand its action for a long time. If, then, you introduce some fat or other into a tissue, harden it with osmic acid and corrode the tissue with eau de Javelle, you will obtain a mould in osmium-blackened and hardened fat, of the spaces you had filled with the fat introduced.

The method may be of much use in certain special researches, such as those on the choroid, iris, and pigmented organs. I recommend the reader to carefully study the article, which does not well bear abstracting. A good abstract will be found in *Journ. Roy. Mic. Soc.*, 1879, p. 610, with plate.

Decalcification and Desilicification.

572. **Decalcification.**—In order to obtain the best results, it is important to employ only material that has been *duly fixed and hardened secundum artem;* and it is well not to put too much confidence in reagents that are said to have the property of hardening and decalcifying fresh material at the same time (FISH, *Ref. Handb. Med. Sci.*, Supp., p. 425.)

573. **Decalcification of Bone.**—I take the following historical sketch from Busch's article "On the Technique of the Histology of Bone" (*Arch. f. mik. Anat.*, xiv, 1877, p. 481; see also the paper of HAUG, in *Zeit. f. wiss. Mik.*, viii, i, 1891, p. 1).

The most widely used agent for decalcification is *hydrochloric* acid. Its action is rapid, even when very dilute, but it has the disadvantage of causing serious swelling of the tissues. To remedy this, chromic acid may be combined with it, or alcohol may be added to it. Or a 3 per cent. solution of the acid may be taken and have dissolved in it 10 to 15 per cent. of common salt. Or (Waldeyer) to a $\frac{1}{1000}$ per cent. solution of *chloride of palladium* may be added $\frac{1}{10}$ of its volume of HCl.

Chromic acid is also much used, but has a very weak decalcifying action and a strong shrinking action on tissues. For this latter reason it can never be used in solutions of more than 1 per cent. strength, and for delicate structures much lower strengths must be taken.

Phosphoric acid has been recommended for young bones.

Acetic, lactic, and *pyroligneous acids* have considerable decalcifying power, but cause great swelling. *Picric acid* has a very slow action, and is only suitable for very small structures.

See further under the head of "Bone."

574. Nitric Acid (Busch, l. c.).—To all other agents Busch prefers nitric acid, which causes no swelling and acts most efficaciously, whilst at the same time it does not injuriously attack tissue-elements.

One volume of chemically pure nitric acid of sp. gr. 1·25 is diluted with 10 vols. water. It may be used of this strength for very large and tough bones; for young bones it may be diluted down to 1 per cent.

Fresh bones are first laid for three days in 95 per cent. alcohol; they are then placed in the nitric acid, *which is changed daily,* for eight or ten days. They must be *removed as soon as* the decalcification is complete, or else they will become stained yellow. When removed they are washed for one or two hours in running water and placed in 95 per cent. alcohol. This is changed after a few days for fresh alcohol.

Young and fœtal bones may be placed in the first instance in a mixture containing 1 per cent. bichromate of potash and $\frac{1}{10}$ per cent. chromic acid, and decalcified with nitric acid of 1 to 2 per cent., to which may be added a small quantity of chromic acid ($\frac{1}{10}$ per cent.) or chromate of potash (1 per cent.). By putting them afterwards into alcohol the well-known green stain is obtained.

575. Nitric Acid and Alcohol.—3 per cent. of nitric acid in 70 per cent. alcohol. Soak specimens for several days or weeks. I do not know who first recommended this admirable medium. Pure nitric acid, even if weak, readily exercises a gelatinising action on bone; whilst the addition of alcohol (or of *alum*) counteracts this action (Fish, *Ref. Handb. Med. Sci.,* Supp., p. 425).

THOMA (*Zeit. f. wiss. Mik.*, viii, 2, 1891, p. 191) gives the following method.—Take 5 vols. of 95 per cent. alcohol and 1 vol. pure concentrated nitric acid. Leave bones in this mixture, changing the liquid every two or three days, until thoroughly decalcified, which should happen even with large bones in two or three weeks. Wash out until every trace of acid is removed (*i. e.* for some days after no acid reaction is obtained with litmus paper) in 95 per cent. alcohol containing an excess of precipitated chalk. This may take eight to fourteen days, after which the tissues will stain well and may be treated as desired.

576. Nitric Acid and Alum (GAGE, quoted from FISH, l. c.).—A saturated aqueous solution of alum is diluted with an equal volume of water, and to each 100 c.c. of the dilute solution is added 5 c.c. of strong nitric acid. Change every two or three days, until the decalcification is complete. For teeth this is said to be, perhaps, a better decalcifier than the alcohol mixture.

577. Hydrochloric Acid (see above, § 573).—RANVIER says that it may be taken of 50 per cent. strength, and then has a very rapid action. To counteract the swelling action of the acid, sodium chloride may be added. Two formulæ of this sort have been given by VON EBNER (see HAUG'S paper quoted in the last section). The first is 100 c.c. cold saturated solution of sodium chloride in water, 100 c.c. water, and 4 c.c. hydrochloric acid. Preparations to be placed in this, and 1 to 2 c.c. hydrochloric acid added daily until they are soft. The second is, 2·5 parts of hydrochloric acid, 500 of alcohol, 100 of water, and 2·5 of sodium chloride. HAUG prefers the proportions of 1·0 to 5·0 of acid, 70 of alcohol, 30 of water, and 0·5 of salt.

578. Hydrochloric Acid and Chromic Acid (BAYERL, *Arch. f. mik. Anat.*, 1885, p. 35).—Equal parts of 3 per cent. chromic acid and 1 per cent. hydrochloric acid. For ossifying cartilage. HAUG recommends equal parts of 1 per cent. hydrochloric acid and 1 per cent. chromic acid (l. c.).

579. Hydrochloric Acid and Nitric Acid (HOPEWELL SMITH, *Journ. Roy. Mic. Soc.*, 1892, p. 433).—Place teeth in 12 parts of 10 per cent. HCl, and after 15 hours add 1·5 parts of HNO_3, and after 48 hours add 1·5 parts more of HNO_3. After 75 to 80 hours remove and wash for half an hour in a solution of 5 grms. of lithium carbonate to an ounce of water.

580. Hydrochloric Acid and Glycerin.—Glycerin, 95; hydrochloric acid, 5. Recommended for softening teeth in SQUIRE'S *Methods and Formulæ*, p. 12.

581. Picric Acid should be taken saturated.

Picro-sulphuric acid should of course be avoided on account of the formation of gypsum.

Picro-nitric or Picro-hydrochloric Acid.—The reader will perhaps reflect that the last two fluids appear likely to be very useful for decalcifications. Mayer points out that the action is very rapid, and that the copiously evolved CO_2 often produces, mechanically, lesions in tissues; so that in many cases in which calcareous structures are concerned chromic acid is to be preferred, the more so as it more effectually hinders any *collapsing* of the structures that might result from the withdrawal of their supporting calcareous elements.

Picric acid fluids are good media, as though their action is slow they preserve tissues well, and leave them in a good state for subsequent staining.

582. Phosphoric Acid.—10 to 15 per cent. (HAUG, l. c., in § 573). Somewhat slow, staining not good.

583. Lactic Acid.—10 per cent. or more. Fairly rapid, preserves well, and may be recommended (HAUG, l. c.).

584. Chromic Acid is employed in strengths of from 0·1 per cent. to 2 per cent., the maceration lasting two or three weeks (in the case of bone). It is better to take the acid weak at first, and increase the strength gradually. In any way the action is extremely slow, and it is therefore better to take one of the mixtures of chromic acid with a more energetic agent.

585. Chromic and Nitric Acid.—Dissolve 15 grms. pure chromic acid in 7 oz. of distilled water, to which 30 minims of nitric acid are afterwards to be added. Macerate for three or four weeks, changing the fluid frequently (Marsh).

FOL takes 70 volumes of 1 per cent. chromic acid, 3 of nitric acid, and 200 of water (*Lehrb.*, p. 112).

FISH takes simply liquid of Perenyi.

It remains to be added that even with the addition of nitric or hydrochloric acid the action is excessively slow, frequently requiring months to be complete.

586. Arsenic Acid.—4 per cent. aqueous solution, used at a temperature of 30° to 40° C. (SQUIRE'S *Methods and Formulæ*, &c., p. 11).

587. Phloroglucin (ANDEER, *Centralbl. f. d. med. Wiss.*, xii, xxxiii, pp. 193, 579; *Intern. Monatschr.*, i, p. 350; *Zeit. f. wiss. Mik.*, 1885, pp. 375, 539; *Journ. Roy. Mic. Soc.*, 1887, p. 504; HAUG, *Zeit. f. wiss. Mik.*, viii, 1, 1891, p. 8; FERRERI, *ibid.*, ix, 2, 1892, p. 236; *Bull. R. Accad. Med. di Roma*, 1892, p. 67).—This is the most recent of the decalcification methods. It has the advantages of being the most rapid of any, and of preserving the tissues very well (with the exception of blood).

Phloroglucin by itself is not a solvent of lime salts; its function in the mixtures given below is so to protect the organic elements of tissues against the action of the mineral acids that these can be used in a much more concentrated form than would be otherwise advisable.

Haug advises the following procedure:—Bring 1 grm. of phloroglucin into 10 c.c. of pure, not fuming nitric acid (1·4 sp. gr.), and warm very slowly and carefully with gentle agitation. There is formed a clear solution of (presumably) a nitrate of phloroglucin. Dilute the solution with 100 c.c. of distilled water, and add 10 c.c. of nitric acid. This gives a solution containing 20 per cent. of acid, which is the proper proportion. More water may be added to the solution, to make it up to 300 c.c., if nitric acid be also added in the proportion given. But the dilution must not be carried beyond this point, in order that the preservative action of the phloroglucin be not overmuch weakened. The process of decalcification in this solution is extremely rapid, and therefore should be carefully watched. Fœtal and young bones become quite soft in half an hour; small pieces of old and hard bones (femur, temporal bone) in a few hours. Teeth take longer, and may require, if time be an object, a solution made with 35 to 45 per cent. of nitric acid. Wash out for two days in running water. The tissues stain well.

The solution may be made with hydrochloric acid instead of nitric acid, 30 per cent. of acid being taken, and 0·5 per cent. of sodium chloride added.

For slow decalcification a 2 to 5 per cent. nitric acid solution may be used, or a mixture containing of phloroglucin 1 part, nitric acid 5, alcohol 70, and distilled water 30 parts.

For the labyrinth, Ferreri advises a mixture containing 1 grm. of phloroglucin, dissolved with the aid of heat in 10 grms. of hydrochloric acid with 100 of water, 200 of 70 per cent. alcohol being added after cooling. The mixture should be changed once a week during thirty to forty days.

Desilicification.

588. Hydrofluoric Acid (Mayer's method, *Zool. Anz.*, 1881, No. 97, p. 593).—The objects from which it is desired to remove siliceous parts are brought in alcohol into a glass vessel coated internally with paraffin (otherwise the glass would be corroded by the acid). Hydrofluoric acid is then added drop by drop (the operator taking great care to avoid the fumes, which attack mucous membranes with great energy). A *Wagnerella borealis* may thus be completely desilicified in a few minutes. Small pieces of siliceous sponges will require a few hours, or at most a day. The tissues do not suffer; and if they have been previously stained with acetic acid carmine the stain does not suffer; at least, this was so in the case of *Wagnerella.*

This dangerous method is best avoided. As regards sponges, I would point out that if well imbedded, good sections may be made from them without previous removal of the spicula. The spicula appear to be cut; probably they break very sharply when touched by the knife. Knives are of course not improved by cutting such sections.

Bleaching.

589. MAYER's **Chlorine Method** (*Mitth. Zool. Stat. Neapel,* ii, 1881, p. 8).—This is a process imagined for the purpose of getting rid of the blackening that often occurs as a consequence of treatment by osmic acid.

The specimens are put into alcohol (either 70 or 90 per cent.). Crystals of chlorate of potash are added until the bottom of the vessel is covered with them. A few drops of concentrated hydrochloric acid are then added by means of a pipette, and mixed in by shaking the vessel as soon as the green colour of the evolving chlorine has begun to show itself. Warm if necessary; but most objects, even large ones, may be bleached in half a day without the employment of heat. The tissues do not suffer.

Instead of hydrochloric acid, nitric acid may be used; in which case the bleaching agent is the freed oxygen, instead of chlorine.

The first method may be used for the purpose of removing pigment from the eyes of insects.

590. MARSH's **Chlorine Method** (*Section Cutting,* p. 89).—Marsh generates chlorine in a small bottle by treating crystals of chlorate of potash with strong HCl, and leads the gas (by means of a piece of glass tubing bent twice at right angles) to the bottom of a bottle containing the sections in water. (See a fig. of the apparatus in *Journ. Roy. Mic. Soc.,* iii, 1880, p. 854.)

591. Chlorine Solution (SARGENT's method).—Hydrochloric acid, 10 drops; chlorate of potash, ½ dr.; water, 1 oz. Soak for a day or two. Wash well.

This method is intended for "bleaching insects;" it will be seen that it is only applicable to the preparation of hard parts, as soft tissues would be destroyed by the solution.

592. Kreasote (POUCHET's method, *Journ. de l'Anat.*, 1876, p. 8, *et. seq.*).—I gather from the paper here quoted that most of the granular animal pigments are soluble in kreasote. Other solvents are mentioned in this paper ("On the Change of Colouration through Nervous Influence"), but this appears to be the only one capable of general histological application.

593. Nitric Acid has a similar action. PARKER (*Bull. Mus. Comp. Zool.*, Cambridge, U.S.A., 1889, p. 173; see *Zeit. f. wiss. Mik.*, viii, 1, 1891, p. 82) says that for eyes of scorpions the usual 5 to 10 per cent. solutions are not strong enough. He treats sections, fixed to the slide with Schällibaum's medium, for about a minute with a solution of up to 50 per cent. of nitric acid in alcohol, or, still better, with a 35 per cent. solution of a mixture of equal parts of nitric and hydrochloric acid in alcohol. To make the solution, the acid should be poured slowly into the alcohol (not *vice versâ*), and the mixture kept cool.

594. Peroxide of Hydrogen (Oxygenated Water) (POUCHET's method, M. DUVAL, *Précis*, &c., p. 234).—Macerate in glycerin to which has been added a little oxygenated water (5 to 6 drops to a watch-glass of glycerin). (Oxygenated water may be procured from perfumers or hair-dressers, by whom it is sold as a hair dye under the name of "Auréoline," "Golden hair-wash," or the like.)

The brownish-green colour communicated to tissues by chromic solutions may be changed to yellow by means of oxygenated water (see § 41).

Osmium preparations may be bleached in the same way (see § 38).

595. Peroxide of Sodium (CARAZZI, *Zool. Anz.*, 444, 1894, p. 135).—Peroxide of sodium, $Na^2 O^2$, is a yellowish powder, which in presence of water gives off oxygen, and communicates an alkaline reaction to the water. With acidified water this alkalinity is not produced. Carazzi advises that a 10 per cent. solution of tartaric or acetic acid be put into a suitable recipient, and a *small* quantity of the peroxide be put at the bottom of the recipient. Alcohol of 70 per cent. is then cautiously poured on to the surface of the liquid, and the objects to be bleached (previously saturated with alcohol) are then put into the supernatant alcohol. Oxygen is given off from the bottom of the liquid, and rises and dissolves in the alcohol, bleaching the objects that have been placed there.

Mineral acids must not be employed, and large quantities of peroxide must not be added to a small quantity of liquid, as the reaction under these conditions is very violent.

596. Sulphurous Acid.—Prof. Gilson writes me that he finds alcoholic solution of sulphurous anhydride (SO^2) very convenient for the rapid decolouration of bichromate objects. A few drops suffice.

597. Eau de Labarraque. Eau de Javelle (see §§ 569, 570).—These are bleaching agents. For the manner of preparing a similar solution see *Journ. de Microgr.*, 1887, p. 154, or *Journ. Roy. Mic. Soc.*, 1887, p. 518. It is, shortly, as follows:—8 parts of caustic soda are dissolved in 100 parts of distilled water, and chlorine is passed through to saturation. During the passage of the chlorine the solution must be surrounded with a mixture of salt and ice, otherwise the temperature rises, and chloride and chlorate of soda are produced. The resulting solution contains 7·45 per cent. of hypochlorite of soda. It is green; and the more effectual the cold, the greener is the colour. The energy of the decolourising action is proportional to the greenness of the solution. Of course the method cannot be used for bleaching soft parts which it is desired to preserve.

598. Chloroform helps to clear strongly pigmented chitin, and combined with nitric acid will decolourise it entirely (see below, in the chapter on Arthropods, Part II).

599. Grenacher's **Mixture for Eyes of Arthropods and other Animals** (*Abh. nat. Ges. Halle-a.-S.*, xvi; *Zeit. f. wiss. Mik.*, 1885, p. 244).

Glycerin	1 part.
80 per cent. alcohol	2 parts.

Mix and add 2 to 3 per cent. of hydrochloric acid.

Pigments dissolve in this fluid, and so doing form a stain which suffices in twelve to twenty-four hours for staining the nuclei of the preparation. You may, if you like, first stain the objects with borax-carmine, and then put them into the liquid, the pigment being washed out more rapidly than the carmine. But the progress of the decolouration must be carefully watched.

CHAPTER XXVII.

EMBRYOLOGICAL METHODS.

600. Artificial Fecundation.—This practice, which affords the readiest means of obtaining the early stages of development of many animals, may be very easily carried out in the case of the Amphibia anura, Teleostea, Cyclostomata, Echinodermata, and many Vermes and Cœlenterata.

In the case of the Amphibia, both the female and the male should be laid open, and the ova should be extracted from the uterus and placed in a watch-glass or dissecting-dish, and treated with water in which the testes, or, better, the vasa deferentia, of the male have been teased.

Females of Teleostea are easily spawned by manipulating the belly with a gentle pressure; and the milt may be obtained from the males in the same way. (It may occasionally be necessary, as in the ease of the Stickleback, to kill the male, and dissect out the testes and tease them.) The spermatozoa of fish, especially those of the Salmonidæ, lose their vitality very rapidly in water; it is therefore advisable to add the milt immediately to the spawned ova, then add a little water, and after a few minutes put the whole into a suitable hatching apparatus with running water.

Artificial fecundation of Invertebrates is easily performed in a similar way. It is sometimes possible to perform the operation under the microscope, and so observe the penetration of the spermatozoon and some of the subsequent phenomena, as has been done by Fol, the Hertwigs, Selenka, and others for the Echinodermata and other forms.

601. Superficial Examination.—The development of some animals, particularly some Invertebrates, may be to a certain extent followed by observations of the living ova under the microscope. This may usefully be done in the case of various Teleosteans, such as the Stickleback, the Perch, *Macropodus*, and several pelagic forms, and with *Chironomus*, *Asellus* *aquaticus*, Ascidians, *Planorbis*, many Cœlenterata, &c. I advise the student to carefully draw the different stages so observed, for such drawings are most important aids to the study of the same stages by the section method.

Some ova of Insecta and Arachnida which are completely opaque under normal conditions become transparent if they are placed in a drop of oil; if care be taken to let their surface be simply impregnated with the oil, the normal course of development is not interfered with (BALBIANI).

602. Preparation of Sections.—Osmic acid, employed either alone or in combination with other reagents, is an excellent fixing agent for small embryos, but not at all a good one for large ones. It causes cellular elements to shrink somewhat, and therefore brings out very clearly the slits that separate germinal layers, and any channels or other cavities that may be in course of formation.

In virtue of its property of blackening fatty matters, myelin amongst them, it is of service in the study of the development of the nervous system.

Chromic acid is indispensable for the study of the external forms of embryos; it brings out elevations and depressions clearly, and preserves admirably the mutual relations of the parts; but it does not always preserve the forms of cells faithfully, and is a hindrance to staining in the mass.

Picric liquids have an action which is the opposite of that of osmic acid; they cause cellular elements to swell somewhat, and thus have a tendency to obliterate spaces that may exist in the tissues. But notwithstanding this defect, the picric compounds, and especially Kleinenberg's picro-sulphuric acid, are amongst the best of embryological fixing agents.

RABL (*Zeit. f. wiss. Mik.*, xi, 2, 1894, p. 165) highly recommends for embryos of Vertebrates, and also for other objects, the following platino-sublimate mixture.

Platinic chloride, 1 per cent. solution	1 vol.
Saturated aqueous sublimate solution	1 „
Distilled water	1 „

This serves for a large number of blastoderms and *young* embryos (Pisces, Amphibia, Aves, Mammalia). *Advanced* embryos of Teleostea ought to be fixed in the *warmed* mixture, in order to avoid rupture of the muscles and shrinkage of the chorda.

Rabl's picro-sublimate mixture has been given § 61. It is recommended especially for somewhat advanced embryos, such as embryo chicks from the third or fourth day, and other embryos of a similar size.

For imbedding, the celloidin-chloroform method of Viallanes gives excellent results, and so does paraffin. This latter is preferable in so far as it lends itself better to the rapid production of series of sections, and allows of the use of the Cambridge Rocking Microtome, the Minot, or the Reinhold-Giltay, which is perhaps the microtome *par excellence* of the embryologist.

As to staining, my eminent fellow-worker, Dr. Henneguy, writing the chapter on embryological methods for the French edition of this work, advised staining in the mass with borax-carmine or alum-carmine (Henneguy's acetic acid formula); or, as an alternative, the staining of sections by Flemming's method. The improvements that have in recent times been worked out in this method give still greater weight to the latter recommendation.

603. Reconstruction of Embryos from Sections.—The study of a series of sections of any highly differentiated organism of unknown structure is so complicated that it is often necessary to have recourse to elaborate methods of geometrical or of plastic reconstruction in order to obtain an idea or a model of the whole. These methods have now been brought to so high a degree of complexity that a volume rather than a paragraph would be necessary to describe them. See Born, "Die Plattenmodellirmethode," in *Arch. f. mik. Anat.*, 1883, p. 591, and *Zeit. f. wiss. Mik.*, v, 4, 1888, p. 433; Strasser, in *Zeit. f. wiss. Mik.*, iii, 2, 1886, p. 179, and iv, 2 and 3, pp. 168 and 330; Kastschenko, in *Zeit. f. wiss. Mik.*, iv, 2 and 3, 1887, pp. 235–6 and 353, and v, 2, 1888, p. 173 (abstracts of all these papers may be found in *Journ. Roy. Mic. Soc.* of the years quoted).

A simple, but in many cases quite efficient plan, has been described by Fol (*Lehrb.*, p. 35) as follows:—Before cutting your sections, you make an outline drawing of your object, under the magnification that you intend to employ for the reconstructed drawing, and in a plane perpendicular to that of the intended sections. For instance, if you intend to make transverse sections of an embryo, begin by making a profile drawing of it, that is, a drawing of the outline of an ideal sagittal section of it. Then make your series of sections, and make drawings of them all under the same magnification as the sagittal drawing. Then trace over your sagittal drawing a series of equidistant parallel lines in positions corresponding to the sections that have been made. If your sections are one hundredth of a millimetre thick, and your drawing be magnified one hundred times, the lines should be one millimetre apart (if you intend to reconstruct the whole of your sections, but the operation may frequently be abridged by only reconstructing say every fifth or every tenth section).

You have now to fill in your outline drawing with details borrowed from the drawings of the sections. You may help yourself greatly in the following way:—A plate of glass, of a size suitable to the intended drawings, is covered with a layer of gelatin, and dried. On this is ruled a series of parallel lines, very close together, and ruled with differently coloured inks, the colours recurring in regular order. The plate is then cut into two unequal parts by a diamond, on a line perpendicular to the coloured lines.

Lay one of the parts of the plate on the outline drawing so that the cut edge covers the line that corresponds to the first section you are going to fill in; then lay the other part of the plate on the drawing of the section in such a position that the limits of the drawing correspond to the same coloured lines that cover the limits of the outline drawing on the other part of the plate already placed.

Trace on the plate that covers the drawing of the section the outline of the internal organs. Lay it against its fellow-plate on the outline drawing, making the coloured lines correspond, and you will easily be able to mark off accurately on the outline drawing a series of dots that correspond in position to the outlines of the internal organs. This operation having been repeated for each of the sections that you desire to bring into your reconstruction, nothing remains but to join your dots by lines, and you will have filled up your outline drawing with a representation of the internal organs in the same plane.

If any reader think this process complicated, he needs but to spend five minutes in trying it with a piece of tracing paper, and will find it to be in reality extremely simple.

Another simple plan is to gum the drawings of the sections on cardboard of a thickness proportional to the thickness of the section and the magnification, cut out all the cavities of the drawing with a knife or fret-saw, and gum all the fretwork thus obtained together, This gives, of course, a model of the object.

In simple cases it may be sufficient to adopt the plan described by SCHAFFER (*Zeit. f. wiss. Mik.*, vii, 3, 1890, p. 342). Careful outlines of the sections to be reconstructed are drawn on tracing-paper with the aid of the camera lucida, superposed, and held up against the light for examination by transparence. EYCLESHEIMER's method of producing orientation lines in celloidin imbedding mass has been described in Chap. X, and some paraffin orientation methods in § 136.

Mammalia.

604. Rabbit—Dissection.—The rabbit may conveniently be taken as a type for this kind of work.

For the study of the early stages the ova must be sought for in the *tubæ* a certain number of hours after copulation. The dehiscence of the follicles takes place about ten hours after the first coitus. The *tubæ* and *cornua* having been dissected out should be allowed to cool, and remain until the muscular contractions have ceased. Then, with the aid of fine scissors or a good scalpel, all the folds of the genital duct are carefully freed from their peritoneal investment.

The tubæ are then (if the ova are still within them, which is the case up to the end of the third day after coition) laid out on a long slip of glass, and slit up longitudinally by means of a pair of fine, sharp scissors. By means of needles and forceps the tubal mucosa is spead out so as to smooth out its folds as much as possible, and is carefully looked over with a strong lens or with a low power of the microscope. When the ova are found, a drop of some "indifferent" liquid is dropped on each, and it is carefully taken up with the point of a scalpel, a cataract needle, or a small pipette. They may be examined in the peritoneal fluid of the mother if the animal has been killed, or in its aqueous humour, or in amniotic liquid, or in blood-serum, or in Kronecker's or other artificial serum.

If you have not been able to find the ova with the lens or the microscope, scrape off the epithelium of the tubal mucosa with a small scalpel, mix it with a little indifferent liquid, and look for the ova under the microscope by transmitted light.

Another method, employed by Kölliker, consists in injecting solution of Müller or weak osmic acid into the oviduct by means of a small syringe, and collecting the liquid that runs out in a series of watch-glasses, in which the ova can very easily be found by the microscope.

The same doe may be made to serve for two observations, at some hours' or days' interval. A longitudinal incision of 8 to 10 centimetres length is made on the median or a lateral line of the abdomen; an assistant keeps the intestines in their place; a ligature is placed at the base of one of the uterine cornua, beneath the neck, and a second ligature around the mesometrium and mesovarium. The ovary, the tuba, and the cornu of that side are then detached with scissors. The abdomen is then closed by means of a few sutures passing through the muscle-layers and the skin. The animals support the operation perfectly well, and the development of the ova of the opposite side is not in the least interfered with. When it is desired to study these the animal may be killed, or may be subjected to a second laparotomy if it be desired to preserve it for ulterior observations.

During the *fourth, fifth,* and *sixth* days after copulation the ova of the rabbit are free in the uterine cornua; they are easily visible to the naked eye, and may be extracted by the same manipulations as those of the tubes. After the sixth day they are at rest in the uterus, but have not yet contracted adhesions with the mucosa, so that they can still be extracted whole. At this stage the parts of the cornua where the ova are lodged are easily distinguishable by their peculiar aspect, the ova forming eminences of the size of a pea. The cornua should be cut up transversely into as many segments as there are eminences, care being taken to have the ova in the centre of the segments. You then fix each segment by means of two pins on the bottom of a dissecting dish, with the mesometrial surface downwards and the ovular eminence upwards. The dissecting dish is then filled up with serum or liquid of Müller, or 0·1 per cent. solution of osmic acid, or Kleinenberg's picro-sulphuric acid, or nitric acid, or acetate of uranium solution. With a small scalpel a longitudinal incision is made on the surface of the ovular eminence, not passing deeper than the muscular layer; the underlying uterine mucosa is then gently dilacerated with two pairs of small forceps, and the ovum set free in the liquid.

From the moment the ova have become adherent to the uterine mucosa they can no longer be extracted whole. The embryo being always situated on the mesometrial surface, the ovular eminence is opened by a *crucial* incision, and the strip of mucosa to which the embryo remains adherent is fixed with pins on the bottom of the dish. ED. V. BENEDEN (see *Arch. de Biol.*, v, fasc. iii, 1885, p. 378) has been able by operating in this way in serum of Kronecker, and keeping the whole at blood temperature, to observe the circulation of the embryo for hours together. (If this be desired to be done, the crucial incision should not be too extended, so as to leave the terminal sinus intact.)

Retterer (*C. R. Soc. de Biol.*, 1887, p. 99) advises that for ova of the seventh day the segment of uterus containing them be opened *on the mesometrial surface,* for at that date no adhesion has yet been contracted with that side. By running in liquid of Kleinenberg by means of a pipette between the ovum and the free surface of the uterus, the ovum may be got away in the shape of a closed vesicle.

605. Rabbit; **Microscopic Preparations.**—In order to make permanent preparations of the different stages of fecundation and segmentation, v. Beneden (*Arch. de Biol.*, i, 1, 1880, p. 149) recommends the following process :—The living ovum is brought into a drop of 1 per cent. osmic acid on a slide, and thence into solution of Müller (or bichromate of ammonia or solution of Kleinenberg). After an hour the liquid is changed, and the whole is put into a moist chamber, where it remains for two or three days. It is then treated with glycerin of gradually increasing strength, and at last mounted in pure glycerin acidified with formic acid. Ova may be stained with Beale's carmine or picro-carmine, after removal from the osmic acid and careful washing.

In order to bring out the outlines of blastoderm-cells the living ovum may be brought into one third per cent. solution of nitrate of silver. After remaining there for half a minute to two minutes, according to the age of the vesicle, it is brought into pure water and exposed to the light. The preparations thus obtained are instructive, but blacken rapidly, and cannot be permanently preserved.

After the end of the third day the blastodermic vesicle can be opened with fine needles, and the blastoderm washed, stained, and mounted in glycerin or balsam ; v. Beneden has also obtained good preparations by means of chloride of gold.

For embryonic areas and more advanced embryos Kölliker recommends putting the ovum into 0·5 per cent. solution of osmic acid until it has taken on a somewhat dark tint, which happens in about an hour, and then treating it with successive alcohols for several hours. If the ovum be adherent to the uterine mucosa the portion of the membrane to which it is fixed should be left, stretched out with pins, in 0·1 per cent. solution of osmic acid for from four to six hours. The blastodermic vesicle can then easily be removed, and immersed for a few hours more in 0·5 per cent. solution of osmic acid, and finally be brought into alcohol. For sections Kölliker fixes with osmic acid. v. BENEDEN treats the ova for twenty-four hours with 1 per cent. solution of chromic acid, then washes well, and brings them through successive alcohols. Chromic acid has the advantage of hardening thoroughly the vesicle, and maintaining at the same time the epiblast-cells perfectly adherent to the zona pellucida. v. Beneden also recommends the liquid of Kleinenberg. HENNEGUY writes that he frequently employs it for embryonic areas and embryos of various ages, always with excellent results. Fol's modification of the liquid of Flemming, and Ranvier and Vignal's osmic acid and alcohol mixture (§ 37), also give excellent results. For staining, Henneguy recommends borax-carmine or Delafield's hæmatoxylin for small embryos; for large ones he found that his acetic acid alum-carmine was the only reagent that would give a good stain in the mass. I think Carmalum is now indicated.

For sections, pure paraffin. Cut in series and mount in balsam.

PIERSOL (*Zeit. f. wiss. Zool.*, xlvii, 2, 1888, p. 155) has been lately using for fixation either Kleinenberg's solution or, for young stages, Altmann's 3 per cent. nitric acid. Staining and cutting as above.

See also WEYSSE, *Proc. Amer. Acad. Arts and Sci.*, 1894, p. 285 (blastodermic vesicle of *Sus scrofa*) and SOBOTTA *Arch. f. mik. Anat.*, xlv, 1895, p. 15 (fecundation and segmentation of the ovum of the mouse; fixation in Flemming's weak mixture, sections stained with Benda's iron hæmatoxylin).

Aves.

606. Superficial Examination.—Excellent instructions on this head are given in FOSTER and BALFOUR'S *Elements of Embryology*, to which, as it is certain to be in the student's hands, he may be referred. What follows here is given merely as being of more recent publication.

If it be desired to observe a living embryo by transmitted light, the egg should be opened under salt solution, as described below. A little of the white is then removed through the window, the egg is lifted out of the liquid, and a ring of gummed paper is placed on the yolk so as to surround the embryonic area. As soon as the paper adheres to the vitelline membrane, which will be in a few minutes, a circular incision is made in the blastoderm outside the paper ring. The egg is put back into the salt solution, and the paper ring removed, carrying with it the vitelline membrane and the blastoderm, which may then be brought into a watch-glass or on to a slide and examined under the microscope (DUVAL).

607. **Gerlach's Window Method** (*Nature*, 1886, p. 497; *Journ. Roy. Mic. Soc.*, 1886, p. 359).—Remove with scissors the shell from the small end of the egg; take out a little white by means of a pipette; the blastoderm will become placed underneath the window thus made, and the white that has been taken out may be replaced on it. Paint the margins of the window with gum mucilage, and build up on the gum a little circular wall of cotton wool; place on it a small watch-glass (or circular cover-glass), and ring it with gum. When the gum is dry, the cover is further fixed in its place by means of collodion and amber varnish, and the egg is put back in its normal position in the incubator. The progress of the development may be followed up to the fifth day through the window.

A description of further developments of this method, with figures of special apparatus, will be found in *Anat. Anz.*, ii, 1887, pp. 583, 609; see also *Zeit. f. wiss. Mik.*, iv, 3, 1887, p. 369.

608. **Preparation.**—During the first twenty-four hours of incubation it is extremely difficult to separate the blastoderm from the yolk, and they should be fixed and hardened together. In later stages, when the embryo is conspicuous, the blastoderm can easily be separated from the yolk, which is very advantageous. To open the egg, lay it on its side and break the shell at the broad end by means of a sharp rap; then carefully remove the shell bit by bit by breaking it away with forceps, working away from the broad end until the blastoderm is exposed. The egg should be opened in salt solution, then lifted up a little, so as to have the blastoderm above the surface of the liquid; the blastoderm is then treated with some fixing solution dropped on it from a pipette (1 per cent. solution of osmic acid, or Ranvier and Vignal's osmic acid and alcohol mixture, iodised serum, solution of Kleinenberg, 10 per cent. nitric acid, &c.). By keeping the upper end of the pipette closed, and the lower end in contact with

the liquid on the blastoderm, the blastoderm may be kept well immersed for a few minutes, and should then be found to be sufficiently fixed to be excised. (Of course, if you prefer it, you can open the egg in a bath of any fixing liquid [10 per cent. nitric acid being convenient for this purpose] of such a depth as to cover the yolk; and having exposed the blastoderm, leave it till fixed [fifteen to twenty minutes]; but I think the procedure above described will generally be found more convenient.)

The egg is put back into the salt solution, and a circular incision made round the embryonic area. The blastoderm may then be floated out and got into a watch-glass, in which it may be examined, or may be brought into a hardening liquid.

Before putting it into the hardening fluid, the portion of vitelline membrane that covers the blastoderm should be removed with forceps and shaking.

Fixation in 10 per cent. nitric acid has the advantage of greatly facilitating the separation of the blastoderm. The acid should be allowed to act for ten minutes, after which it is well to bring the preparation into 2 per cent. solution of alum (*cf.* Hofmann, *Ziet. f. wiss. Mik.*, x, 4, 1893, p. 485).

In order to counteract the turning up of the edges of the blastoderm that generally happens during the process of hardening, it is well to get the blastoderm spread out on the *convex* surface of a watch-glass, and leave it so during the hardening.

For hardening, Foster and Balfour recommend solution of Kleinenberg for five hours, followed by alcohol. Or chromic acid, a solution of 0·1 per cent. for twenty-four hours, followed by a solution of 0·3 per cent. for twenty-four hours more, then by 70 per cent. alcohol for a day, 90 per cent. alcohol for two days, and lastly absolute alcohol. They also recommend a 0·5 per cent. solution of osmic acid, in which the embryo remains for two hours and a half in the dark, and after washing is brought into absolute alcohol.

Henneguy prefers the osmic acid and alcohol mixture of Ranvier and Vignal, or Flemming's mixture followed by successive alcohols.

Staining and imbedding may be performed by the usual methods.

Up to about the fiftieth hour embryos may be mounted entire, in glycerin or balsam.

609. M. Duval's **Orientation Method** (*Ann. d. Sc. nat. Zool.*, 1885).—In the early stages of the development of the ova of Aves, before the appearance of the primitive streak, it is difficult to obtain a correct orientation of the hardened cicatricula, so as to be able to make sections in any desired direction. Duval, starting from the fact that during incubation the embryo is almost always found to be lying on the yolk in such a position that the big end of the egg is to the left, and the little end to the right of it, marks the position of the blastoderm in the following way.

With a strip of paper 5 millimetres wide and 50 millimetres long you construct a sort of triangular bottomless box. You lay this on the yolk, enclosing the cicatricula in such a position that the base of the triangle corresponds to what will be the anterior region of the embryo, and its apex to the posterior region; that is to say, if the big end of the egg is to your left, the apex of the triangle will point towards you. You now, by means of a pipette, fill the paper triangle with 0·3 solution of osmic acid. As soon as the preparation begins to darken you put the whole egg into weak chromic acid, remove the white, and put the rest into clean chromic acid solution for several days. After hardening you will find on the surface of the yolk a black triangular area, which encloses the cicatricula and marks its position; you cut out this area with scissors and a scalpel, and complete the hardening with chromic acid and alcohol.

See also the critique and modification of this method by Kionka, *Anat. Hefte*, x, p. 391; *Zeit. f. wiss. Mik.*, xi, 2, 1894, p. 250 (stain with borax carmine, adding to the acid alcohol used for washing out a few drops of aqueous solution of Orange G, which stains the vitellus yellow).

See also the method of Hirota, *Journ. Roy. Mic. Soc.*, 1895, p. 118.

610. Vialleton's **Method** (*Anat. Anzeig.*, vii, 1892, pp. 624—627; *Journ. Roy. Mic. Soc.*, 1892, p. 889).—Egg opened in salt solution, blastoderm excised and removed to a glass plate, then treated with 1 per cent. nitrate of silver solution, washed with water, and put into 70 per cent. alcohol for six to twelve hours in the dark. Borax-carmine, alcohol, dammar.

Reptilia.

611 General Directions.—The methods described above for the embryology of birds are applicable to the embryology of reptiles. During the early stages the blastoderm should be hardened *in situ* on the yolk; later the embryo can be isolated, and treated separately with Kleinenberg's solution and alcohol (STRAHL, *Arch. f. Anat. u. Phys.*, 1881, p. 123).

612. PERÉNYI'S **Method** (*Zool. Anzeig.*, 274, 1888, p. 139, and 276, p. 196).—Fix in the following special mixture :

3 parts 20 per cent. nitric acid.
3 parts 1 per cent. chromic acid.
4 parts absolute alcohol.

For embryos of Lacerta. Fix for twenty minutes. Wash out for an hour with 70 per cent. alcohol, and then with strong alcohol. Stain with Delafield's hæmatoxylin, and treat the stained material for three to five minutes with 1 per cent. chromic acid.

613. See also KUPFFER'S **Method** (*Arch. f. Anat. und Entwick.*, 1882, p. 4). SARASIN'S **Method** (SEMPER'S *Arbeiten*, 1883, p. 159), and MITSUKURI (*Journ. Roy. Mic. Soc.*, 1894, p. 750).

Amphibia.

614. Preliminary.—In order to prepare the ova of Amphibia for section cutting, it is essential to begin by removing their thick coats of albumen. This may be done by putting them for two or three days into 1 per cent. solution of chromic acid, and shaking well; but ova thus treated are very brittle, and do not afford good sections. A better method is that described by WHITMAN (*Amer. Natural.*, xxii, 1888, p. 857), and by BLOCHMANN (*Zool. Anz.*, 1889, p. 269). Whitman puts the fixed eggs into a 10 per cent. solution of sodium hypochlorite diluted with 5 to 6 volumes of water, and leaves them there till they can be shaken free, which happens (for *Necturus*) in a few minutes. Blochmann takes *eau de Javelle* (potassium hypochlorite), and dilutes it with 3 to 4 volumes of water, and agitates the eggs previously fixed with solution of Flemming, for fifteen to thirty minutes in it. The ova are afterwards preserved in alcohol in the usual way. Some other means of attaining the same end are given in the following paragraphs.

615. Axolotl.—The ova are easier to prepare than those of the Anura, because the yoke is separated from the albuminous layer by a wide space filled with a liquid that is not coagulated by reagents. Put the eggs for a few hours into picro-sulphuric acid, then pierce the inner chorion with fine scissors or needles, and gently press out the ovum. Harden in alcohol.

616. Triton (SCOTT and OSBORN, *Quart. Journ. Mic. Soc.*, 1879, p. 449).—The albumen is here present in the form of several concentric coats, which are very delicate. Incise each of them separately with fine scissors, turn out the ovum, and fix it. Solution of Kleinenberg is the reagent that gives the best results.

617. Triton (HERTWIG, *Jen. Zeit. f. Naturw.*, 1881-2, p. 291).—Put the eggs into a mixture of equal parts of 2 per cent. acetic acid and 0·5 per cent. chromic acid. After ten hours incise the membranes, opening one end of the inner chorion, and turn out the embryos and bring them into successive alcohols.

618. Salamandra (RABL, *Morphol. Jahrb.*, xii, 2, 1886, p. 252).—For his more recent methods see § 602.

619. Rana (O. HERTWIG, *Jen. Zeit. f. Naturw.*, xvi, 1883, p. 249).—The ova are thrown into nearly boiling water (90° to 96° C.) for five to ten minutes. The albuminous envelope of the ovum is then cut open, and the ovum extracted under water. The ova are then brought into 0·5 per cent. osmic acid, or into alcohol of 70, 80, and 90 per cent. Chromic acid makes ova brittle; they ought not to remain in it for more than twelve hours. Chromic acid destroys or attacks the pigment of the ova, whilst alcohol preserves it, which is frequently important for the study of the germinal layers.

MORGAN (*Amer. Nat.*, xxv, 1891, p. 759; *Journ. Roy. Mic. Soc.*, 1892, p. 284) has the following. During the periods in which it is difficult or impossible to remove the inner jelly-membrane the eggs can be freed as follows:—Each egg is cut out with scissors from the general jelly-mass, and put for from one to twelve hours into saturated solution of picric acid in 35 per cent. alcohol containing "the same amount of sulphuric acid as in Kleinenberg's solution." Wash for several hours in several changes of alcohol, beginning with 35 per cent., and increasing the strength gradually up to 70 per cent. About the second day in the 70 per cent. alcohol the inner membrane begins to swell, and on the third or fourth day may be pierced by a needle, and the egg removed and placed in 80 per cent. alcohol (see also WHITMAN, *Meth. of Research*, p. 156; and SCHULTZE, *Zeit. f. wiss. Zool.*, v, 1887, p. 177).

620. Sulphate of Copper Hardening Liquid. — I take from the *Lehrbuch* of FOL (p. 106) the following formula, which was first published by REMAK, then modified by GOETTE, and is said to be useful for hardening the ova of Amphibia:

2 per cent. solution of sulphate of copper . .	50 c.c.
Alcohol of 25 per cent.	50 „
Rectified wood vinegar	35 drops.

621. Other Methods.—ROUX, **Study of Cleavage Cells (Cytropism of the Ovum)**, see *Arch. f. Entwickelungs-mechanik*, i, p. 44; *Amer. Natural.*, xxix, 1895, p. 511; *Journ. Roy. Mic. Soc.*, 1895, p. 598. **Production of Hemiembryos of the Frog, &c.**, *Anat. Anz.*, ix, 8, 1894, p. 248, and 9, p. 265. BORN, *Arch. f. mik. Anat.*, xliii, 1894, p. 1; RABL, *Zeit. f. wiss. Mik.*, xi, 2, 1894, p. 165 (his fixing methods have been given).

Pisces.

622. Teleostea in General.—The ova of many of the bony fishes can be studied by transmitted light in the living state; but those of the Salmonidæ must be hardened and removed from their envelopes for the study of the external forms of the embryo.

To this end the ova may be put for a few minutes into water containing 1 to 2 per cent. of acetic acid, and thence into 1 per cent. chromic acid. After three days the capsule of the ovum may be opened at the side opposite to the embryo, and be removed with fine forceps. The ovum is put for twenty-four hours into distilled water, and then into successive alcohols. Embryos thus prepared show no deformation, and their histological elements are fairly well preserved. But the vitellus rapidly becomes excessively hard and brittle, so as greatly to interfere with section cutting.

The following processes give good results as regards section cutting.

Put the ova for a few minutes into 1 per cent. osmic acid; as soon as they have taken on a light brown colour bring them into Müller's solution. Open them therein with fine scissors—the vitellus, which immediately coagulates on contact with air, dissolves, on the contrary, in Müller's solution —and the germ and cortical layer can be extracted from the capsule of the ovum. They should be left in clean Müller's solution for a few days, then washed with water for twenty-four hours, and brought through successive alcohols.

Another method (Henneguy) is as follows:—The ova are fixed in solution of Kleinenberg containing 10 per cent. of acetic acid. After ten minutes they are opened in water containing 10 per cent. of acetic acid, which dissolves the vitellus. The embryos are put for a few hours into pure solution of Kleinenberg, and are then brought through alcohol of gradually increasing strength.

623. Kollmann's **Fixative** (Kollmann, *Arch. f. Anat. u. Phys.*, 1885, p. 296).

Bichromate of potash	5 per 100.
Chromic acid	2 ,,
Concentrated nitric acid	2 ,,

For ova of Teleostea. Fix for twelve hours, wash with water for twelve hours, then remove the chorion, and put the ova into 70 per cent. alcohol.

624. Rabl's **Method** (see § 602).

625. Perényi's **Method** (see § 50).

626. Kowalewsky's **Method** (see *Zeit. f. wiss. Zool.*, xliii, 1886, p. 434).

627. Boyer's **Methods.**—See *Bull. Mus. Comp. Zool., Harvard*, xxiii, 1892, p. 93; *Journ. Roy. Mic. Soc.*, 1892, p. 699.

628. Rabl-Rückhard's **Method** (*Arch. f. Anat. u. Entw.*, 1882, p. 67). —Fix in 10 per cent. nitric acid for fifteen minutes. Remove the membranes to avoid deformation of the embryos, and put the ova back into the acid for an hour. Wash out in 1 to 2 per cent. solution of alum for an hour, and harden in alcohol.

Modification of this method by Goronowitsch (see *Morph. Jahrb.*, x, 1884, p. 381).

629. Pelagic Fish Ova (Whitman's method; *Amer. Natural.*, xvii, 1883, pp. 1204–5; *Journ. Roy. Mic. Soc.* [N.S.], iii, 1883, p. 912, and *Methods of Research*, &c., p. 152).—Fix by treatment first for five to ten minutes with a mixture of equal parts of sea water and ½ per cent. osmic acid solution, and then for one or two days with a modified Merkel's solution (due to Eisig), consisting of equal parts of 0·25 per cent. platinum chloride and 1 per cent. chromic acid. Prick the membrane before transferring to alcohol. Whitman found that the usual Merkel's fluid caused maceration of the embryonic portion of the egg. Picro-sulphuric acid causes the embryonic cells to swell, and in many cases to become completely disorganised. The osmic acid treatment is necessary in the case of segmenting ova because the Merkel's fluid does not kill rapidly enough, so that eggs placed in it may even pass through one or two stages of cleavage before dying. This fluid arrests the process of blackening by the osmium, or will even bleach the objects if blackening has set in. See also AGASSIZ and WHITMAN, in *Proc. Amer. Acad. Arts and Sciences*, xx, 1884. For later stages the authors recommend the method of Perényi. And see the experiments detailed by COLLINGE, *Ann. and Mag. Nat. Hist.*, x, 1892, p. 228; *Journ. Roy. Mic. Soc.*, 1892, p. 883.

Tunicata.

630. **Distaplia**.—DAVIDOFF (*Mitth. Zool. Stat. Neapel*, ix, 1, 1889, p. 118) has some important observations on the fixation of the ova of *D. magnilarva.* The best reagent is a mixture of 3 parts of saturated solution of corrosive sublimate and 1 of glacial acetic acid. The ova to remain in it for from half an hour to an hour, and be then washed for a few minutes in water and brought through successive alcohols. Another reagent, almost as good, consists of 3 parts of saturated solution of picric acid and 1 of glacial acetic acid, the objects to remain in it for three to four hours, and then be brought into 70 per cent. alcohol.

631. **Amarœcium** (MAURICE and SCHULGIN, *Ann. Sci. Nat. Zool.*, xvii, 1884).—Stain in borax-carmine, wash out, and stain for fifteen to twenty hours in very weak solution of *bleu de Lyon* in 70 per cent. alcohol with a few drops of acetic acid. In sections the epiblast and hypoblast appear chiefly blue, the mesoblast cells, on the contrary, appearing almost entirely red.

632. **Test-Cells of Ascidians** (MORGAN, *Journ. of Morphol.*, iv, 1890, p. 195).—Tease fresh ovaries in very weak osmic acid, wash in distilled water, treat for half an hour with 1 per cent. silver nitrate, wash for half an hour in 2 per cent. acetic acid, and reduce in sunlight. Imbed in paraffin. By this process the *limits* of the follicle cells are demonstrated.

Mollusca.

633. **Cephalopoda** (USSOW, *Arch. de Biol.*, ii, 1881, p. 582). —Segmenting ova are placed, without removal of the mem-

branes, in 2 per cent. solution of chromic acid for two minutes, and then in distilled water, to which a little acetic acid (one drop to a watch-glassful) has been added, for two minutes. If an incision be now made into the egg-membrane the yolk flows away and the blastoderm remains; if any yolk still cling to it, it may be removed by pouring away the water and adding more.

WATASÉ (*Journ. of Morphol.*, iv, 1891, p. 249; *Journ. Roy. Mic. Soc.*, 1892, p. 152) kills the ova in the macerating mixture of the Hertwigs (§ 553), and as soon as the blastoderm turns white and opaque removes it under dilute glycerin. Treatment with liquid of Perényi is recommended for surface views of cleavage.

634. **Gastropoda** (HENNEGUY).—Ova of Helix may be fixed for from four to six hours in Mayer's picro-nitric solution (§ 76). The carbonate of lime that encrusts the external membrane is thus dissolved, and the albuminous coat of the egg is coagulated. The egg is opened with needles, the albumen comes away in bits, and the embryo can be removed. Treat with successive alcohols, and imbed in paraffin.

635. **Limax** (early stages) (MARK, *Bull. Mus. Comp. Zool., Harvard Coll.*, vi, 1881).—The ova are treated with acetic acid of 1 to 2 per cent. for four or more hours. The two external membranes are incised with fine scissors, and the egg squeezed out in its albumen membrane. This is dissected off on a slide, the egg is separated from the albumen, stained, and mounted in glycerin.

For later stages, or for making sections, osmic acid is used instead of acetic acid, and the egg is hardened with its albuminous coats.

MISS A. HENCHMAN (*Bull. Mus. Comp. Zool., Harvard*, xx, 1890, p. 171; *Journ. Roy. Mic. Soc.*, 1891, p. 274; *Zeit. f. wiss. Mik.*, viii, 2, 1891, p. 216) finds that the best way of obtaining embryos is to keep some twenty-five or thirty adults (of *L. maximus*) in a large tin pail with a cover perforated with small holes. They should be fed on cabbage, and the vessel kept very clean. Eggs are generally found in the morning in bunches of thirty to forty. As they are more abundant in the early stages of confinement it is better to obtain a few slugs often than many at once. In a moderately warm room hatching occurs between the twenty-second and twenty-seventh day. The eggs must be carefully protected from desiccation.

Kill with 0·33 per cent. chromic acid, or with liquid of Perényi. It is best to remove only the outer envelope before putting into the chromic acid, the inner membrane being removed after two or three minutes therein. Where Perényi is used the membranes must be removed first, as the albumen will else coagulate in such a way as to prevent the removal of the embryos.

See also SCHMIDT, *Studien zur Entwickelungsgesch. d. Pulmonaten*, Dorpat, 1891; and WASHBURN, *Amer. Natural.*, xxviii, 1894, p. 528; or *Journ. Roy. Mic. Soc.*, 1894, p. 531. The paper of KOFOID in *Bull. Mus. Comp. Zool., Harvard Coll.*, xxvii, 2, p. 35, contains a detailed account of the treatment appropriate to the ova of *Agriolimax agrestis*, which are smaller than those of *Limax maximus*, studied by Miss Henchman. See also the abstract of these methods in *Journ. Roy. Mic. Soc.*, 1895, p. 701.

636. CHITON, see METCALF, *Stud. Biol. Lab. Johns Hopkins Univ.*, v, 1893, p. 251, or *Journ. Roy. Mic. Soc.*, 1894, p. 531.

Arthropoda.

637. Fixation of Ova.—In most cases the ova of Arthropods are fixed by heat in a more satisfactory way than by any other means. This may be followed either by alcohol or some watery hardening agent. If it be desired to avoid heating, picro-sulphuric acid or liquid of Perényi may be tried.

638. Removal of Membranes.—This is frequently very difficult, and it may often be advisable not to attempt to remove them, but to soften them with *eau de Javelle* or *eau de Labarraque* (see the methods of Looss and LIST).

MORGAN (*Amer. Natural.*, xxii, 1888, p. 357; *Zeit. f. wiss. Mik.*, vi, 1, 1889, p. 69) recommends (for the ova of *Periplaneta*) *eau de Labarraque* diluted with five to eight volumes of water, and slightly warmed. Thus used, it will soften the chitin membranes sufficiently in thirty to sixty minutes if employed before fixing. Fixed ova take longer. The fluid must, of course, not be allowed to penetrate into the interior of the ovum.

639. HENKING'S **Methods**.—For the whole subject of the technique of the embryology of Insecta see an elaborate paper by this specialist in *Zeit. f. wiss. Mik.*, viii, 2, 1891, p. 156. Henking agrees with other workers at this subject, that in the majority of cases heat is the only available fixing agent that will give fair results. He kills ova by plunging them into hot water, or by pouring hot water on to them in a watch-glass, and then removing into 70 per cent. alcohol. But, as might be expected, he finds that the preservation of structures by this method is far from being perfectly satisfactory, cell-contours being not at all sharply brought out by it, and achromatic cell-structures being but imperfectly preserved. He finds that in some cases ova may be fixed with liquid of Flemming, which, as may be supposed, gives incomparably better results in these respects. Suitable ova may be put into liquid of Flemming (Henking does not say which formula) for half an hour, then for two hours into the same diluted with three volumes of water, then treated with alcohol as usual. Boveri's picro-acetic acid was found not to penetrate the membranes.

Henking thinks that eau de Javelle for softening membranes is best avoided. Membranes should either be dissected away or left *in situ*, and cut with the rest of the egg, according to the nature of the case. The great obstacle to section cutting is the brittleness of the yolk. This difficulty may be overcome as follows :—After fixing and treating with alcohol, prick the chorion and stain with borax-carmine. Put the stained ova for twelve hours into a mixture containing 20 c.c. of 70 per cent. alcohol, one drop of concentrated hydrochloric acid, and a knife-pointful of pepsin (it is not necessary that all the pepsin should be dissolved). The ova may then be treated with alcohol, oil of bergamot, and paraffin, and (with some exceptions, amongst which is *Bombyx mori*) will be found to cut without crumbling.

The contents of fresh ova may conveniently be studied by means of the following fluid :

Distilled water	80 c.c.
Glycerin	16 „
Formic acid	3 „
1 per cent. osmic acid . . .	1 „
Dahlia	0·04 grm.

The eggs are simply teased in a drop of the liquid, and a cover-glass put on. If it be desired to preserve the preparation, nothing more is necessary than to lute the cover-glass.

640. Lepidoptera (Bobretzky, *Zeit. f. wiss. Zool.*, 1879, p. 198).—Ova (of *Pieris cratægi* and *Porthesia chrysorrhœa*) are slightly warmed in water and put for sixteen to twenty hours into 0·5 per cent. chromic acid. The membranes can then be removed, and the ova brought for a few hours into absolute alcohol, stained with carmine, and cut.

641. Blattida (Patten, *Quart. Journ. Mic. Sci.*, 1884, p. 549). —The ova or larvæ are placed in cold water, which is gradually raised to 80° C. You leave off heating as soon as the ova have become hard and white. Pass very gradually through successive alcohols, beginning with 20 per cent.; stain with Kleinenberg's hæmatoxylin or Mayer's cochineal (only alcoholic stains will traverse the chorion). The ova may remain in the hæmatoxylin for five or six days, and be washed out in alcohol containing one drop of HCl per 20 grms., in which they should remain for several days, and then be soaked in pure alcohol until they have regained their violet colour. Penetrate with benzol and imbed in paraffin.

Wheeler (*Journ. of Morph.*, iii, 1889, p. 292; *Journ. Roy. Mic. Soc.*, 1890, p. 250) dissects out ovarian ova in salt solution and fixes in liquid of Perényi (fifteen minutes), then treats with alcohol, and stains with borax-carmine. Laid eggs may be killed by Patten's method. After heating, the two lips of the crista of the capsule may be separated with fine forceps and pieces of the walls torn away, and the eggs pushed out of the compartments formed by their choria and hardened as desired. Good results are also obtained by heating to 80° C. for ten minutes in liquid of Kleinenberg, and preserving in 70 per cent. alcohol. This causes the envelopes to dilate and stand off from the surface of the egg, so that they can easily be dissected away.

Cholodkowsky (*Mem. Acad. Imp. St. Petersburg*, xxxviii, 1891; *Zeit. f. wiss. Mik.*, ix, 1, 1892, p. 80) recommends cutting off the ends of the cocoons and fixing for twelve hours in liquid of Perényi, or for a few minutes in a solution of 1 part iodine, 1 part iodine of potassium, and 300 parts water, heated to boiling-point.

Heymons (*Zeit. f. wiss. Zool.*, liii, 1892, p. 434; *Zeit. f. wiss. mik.*, ix, 3, 1893, p. 343) finds that Cholodkowsky's methods are good for the study of general relations of parts, but not satisfactory for the preservation of delicate detail. For young embryos it is better to incise the cocoon at the end by which it inheres in the body of the mother, bring it for two minutes into water heated to 90° C., and open in Flemming, in which the embryo should be dissected out.

642. Diptera (Henking, *Zeit. f. wiss. Zool.*, xlvi, 1888, p. 289; *Zeit. f. wiss. Mik.*, 1889, p. 59).—Ova still contained within the fly may be fixed by plunging the animal for some time into boiling water, then dissecting out and bringing them into 70 per cent. alcohol. Laid eggs may have boiling water poured over them, or be put into solution of Flemming in a test-tube which is plunged into boiling water until the eggs begin to darken (about a minute). Cold solution of Flemming easily causes a certain vacuolisation of the contents of the ova. Open the ova at the larger end, stain with borax-carmine for fifteen to thirty hours, and cut in paraffin.

See also (for *Chironomus*) Ritter, *Zeit. f. wiss. Zool.*, i, 1890, p. 408; *Zeit. f. wiss. Mik.*, viii, 1, 1891, p. 87 (strings of ova fixed with hot 30 per cent. alcohol containing a little sublimate, and stained in the mass by immersion for several days in picro-carmine).

643. Phalangida (Balbiani).—The ova of *Phalangium opilio* are enclosed in a chorion covered with yellow corpuscles which renders them quite opaque. They may be cleared by treating them with water containing a little solution of caustic potash and raised to boiling point. The ova are then laid on blotting-paper, and the chorion is removed by rubbing them gently with a small brush. The vitelline membrane remains intact and transparent, and the embryo may be studied through it.

644. Phalangida (Henking, *Zeit. f. wiss. Mik.*, iii, 4, 1886, pp. 470 *et. seq.*).—Fix with boiling water or "Flemming." Preserve the ova in 90 per cent. alcohol. To open the chorion, bring them back into 70 per cent. alcohol, which causes them to swell up so that the chorion can easily be pierced with needles, and the ovum turned out.

645. Araneina.—See Balfour (*Quart. Journ. Mic. Sci.*, 1880, p. 167); Kishinouye (*Journ. Coll. Sci. Imp. Univ. Japan*, iv, 1891, p. 55; *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 215); Locy (*Bull. Mus. Comp. Zool., Harvard*, xii, 3, 1886; *Zeit. f. wiss. Mik.*, iii, 2, 1886, p. 242).

646. Aphides (Will, *Semper's Arbeiten*, 1883, p. 223).—Sections to be made through the entire animals containing the ova and embryos. The animals are killed in water of 70° C., and brought into alcohol.

647. Astacus (Reichenbach, from *Zeit. f. wiss. Mik.*, 1886, p. 400).—Fix in water gradually warmed to 60° or 70° C. (if the chorion should burst, that is no evil), harden for twenty-four hours in 1 to 2 per cent. bichromate of potash or 0·5 per cent. chromic acid, wash out for the same time in running water, and bring into alcohol. Remove the chorion, remove the embryo from the yoke by means of a sharp knife, and stain with picro-carmine and mount in balsam.

648. Amphipoda (*Orchestia*) Ulianin, *Zeit. f. wiss. Zool.*, xxxv, 1881, p. 441).—Ova in the earliest stages of development were treated for two hours with picro-sulphuric acid (Kleinenberg's formula). This causes the chorion to swell and burst. Ova in later stages, in which the embryo is surrounded by a cuticular membrane, which encloses an albuminous liquid, must have this membrane torn with needles and the albuminous liquid allowed to ooze out before placing in the picro-sulphuric acid.

Vermes.

649. Tænia (v. Beneden, *Arch. de Biol.*, ii, 1881, p. 187).—Ova in which a chitinous membrane has formed around the embryo are impervious to reagents. They may be put on a slide with a drop of some liquid and covered. Then, by withdrawing the liquid by means of blotting-paper, the cover may be made to gradually press on them so as to burst the membranes, and the embryo may then be treated with the usual reagents.

650. Planaria (Iijima, *Zeit. f. wiss. Zool.*, xl, 1884, p. 359).—The capsule containing the ova (of fresh-water Planaria) is opened with needles on a slide, in a drop of 2 per cent. nitric acid. The ova are extracted and covered (the cover being supported by paper, or by wax feet). After half an hour they are treated with successive alcohols under the cover, and finally mounted in glycerin. For sections, the whole of the contents of a capsule is hardened in the mass in 1 per cent. chromic acid and cut together.

651. Lumbricus (Kleinenberg, *Quart. Journ. Mic. Sci.*, 1879, p. 207).—Fix with Kleinenberg's picro-sulphuric acid, or, which is not quite so good, with vapours of osmium, pass through successive alcohols, stain with Kleinenberg's hæmatoxylin, and cut in paraffin.

Wilson (*Journ. of Morph.*, iii, 1889, p. 445; *Journ. Roy.*

Mic. Soc., 1890, p. 402) finds that liquid of Perényi is by far the best fixing reagent, being in most respects superior even to Flemming's. Fix for fifteen to sixty minutes; wash out in 70 per cent. alcohol.

For *Nereis*, see VON WISTINGHAUSEN, *Mitth. Zool. Stat. Neapel*, x, 1891, p. 41; or *Zeit. f. wiss. Mik.*, x, 4, 1893, p. 479.

652. Ascaris.—See the chapter on "Cytological Methods."

Echinodermata, Cœlenterata, and Porifera.

See the paragraphs treating of these groups in the chapter on "Zoological Methods."

For the maturation and fecundation of the ova of the Echinodermata, see also the chapter on "Cytological Methods."

REESE LIBRARY
OF THE
UNIVERSITY
OF CALIFORNIA

CHAPTER XXVIII.

CYTOLOGICAL METHODS.

653. Subjects for Study.—One of the best objects for this purpose is the tail of young larvæ of Amphibia, both Anura and Urodela.

In the *living animal* the epithelial cells and nuclei (in the state of repose) are so transparent as to be invisible in the natural state. They may, however, be brought out by curarising the larva; or, still better, by placing the curarised larva for half an hour in 1 per cent. chloride of sodium solution. Normal larvæ may be used for the study of the active state of the nucleus, but much time is saved by using curare.

Curare.—Dissolve 1 part of curare in 100 parts water, and add 100 parts of glycerin. Of this mixture add from 5 to 10 drops (according to the size of the larva), or even more for large larvæ, to a watch-glassful of water. From half to one hour of immersion is necessary for curarisation. The larvæ need not be left in the solution until they become quite motionless; as soon as their movements have become slow they may be taken out and placed on a slide with blotting-paper. If they be replaced in water they return to the normal state in eight or ten hours, and may be re-curarised several times.

Etherisation.—Three per cent. alcohol, or 3 per cent. ether may be used in a similar way. These reagents cause no obstruction to the processes of cell-division, and are useful, but their action as anæsthetics is inconstant.

Indifferent Media.—One per cent. salt solution, iodised serum, syrup, cold water (+ 1° C.), and warm water (35°—40° C.). The tail may be excised from the living animal and studied for a long time in these media (PEREMESCHKO, *Arch. f. mik. Anat.*, xvi, 1879, p. 437).

Perhaps (FLEMMING, *ibid.*, pp. 304 *et seq.*) the very best subject for these studies is *Salamandra.* The adult offers for study the thin transparent bladder; in the larva the gills and caudal "fin" may be studied in the living state. The gills are difficult to fix in position for observation, and are obscured by pigment. In the fin there is always a spot, near to the hind limbs, that is free from pigment; and on lightly coloured larvæ other such spots may be found on the ventral half of the fin and on the lateral line. On a flat-finned larva it is possible to study these spots with high-power glasses.

The larva may be fixed in a suitable cell, or wrapped in moist blotting-paper, or may be curarised; or the tail may be excised. (It is preferable to cut through the larva close in *front* of the hind limbs.)

Objects for Preparation.—A favorable object for *preparation* is found in the gill-*plates,* delicate laminæ that are to be found attached to the gill-cartilages on the mouth side.

The lungs, parietal peritoneum, and mesentery of the larvæ are also very favorable objects for preparations (see FLEMMING, *Arch. f. mik. Anat.*, xxxv, 1890, p. 275; and xxxvii, 1891, pp. 249 and 685). To prepare the lungs the larva, which should be of not more than 4 cm. length, should be killed by immersion in chromo-aceto-osmic acid, the body-cavity cut into, and the viscera gently drawn out and exposed to the action of the liquid, care being taken not to let the lungs get into folds. After fixation they should be carefully got on to a slide, and a small strip removed from their margins on either side by means of a scalpel, after which the two walls may be separated from each other, and utilised as thin, flat preparations.

Another excellent object is the intestine of the adult, of which sections may be made by the paraffin method, as recommended in the important paper of M. HEIDENHAIN, "Über Kern und Protoplasma," in *Festschr. z.* 50 *jähr. Jubil. d. H. Prof. Geheimr. v. Kölliker* (also in separate reprint), Engelmann, Leipzig, 1892, p. 111. This organ offers for study, besides the large epithelium cells of the intestinal crypts, numberless examples of leucocytes, an extremely favorable object.

Larvæ may be bred from adults kept in confinement, and supplied with a vessel of water, in which they will place the larvæ of their own accord. In May gravid females may be killed and the larvæ extracted. The larvæ must be kept in frequently changed water and fed every day or two. Aquatic worms may be used for feeding them, *e. g. Tubifex rivulorum.*

It is extremely important that they should be fed regularly and abundantly, for, if not, cell-divisions in the tissues become rare, and may even cease altogether.

Adults may easily be kept in a vivarium. The bottom should be covered to a depth of about four inches with mould; a shallow dish (a photographic developing dish is as good as anything) filled with water should be sunk in the mould, and the mould should be covered with moss. The animals should be fed with worms, which they are sure to take during the night (HENNEGUY, *Leçons sur la Cellule,* p. 68).

LÖNNEBERG (see *Zeit. f. wiss. Mik.,* x, 3, 1893, p. 377) recommends the intestinal epithelium of hibernating snails as an object that furnishes an abundance of karyokinetic figures. The snails should be kept for some days in a warm room, their epiphragm should then be removed, and they should be fed for three days with cabbage leaves before being dissected.

Other classical subjects of study will be found mentioned in the following paragraphs.

654. Stains for living Cells.—It is sometimes of the very greatest importance to be able to stain a cell in the living state, even though it be but feebly and imperfectly. See § 213.

655. Study of Fresh and lightly fixed Cells.—It has been rightly pointed out by Flemming that so-called "indifferent" liquids must not be believed to be without action on nuclei. Iodised serum, salt solution, serum, aqueous humour, lymph, better deserve the name of weak hardening agents. Between these, and such energetic hardening agents as Flemming's mixture, come such light fixing agents as picric acid or very dilute acetic acid. These it is whose employment is indicated for the study of fresh isolated cells.

A typical example of this kind of work is as follows:—Tease out a piece of living tissue in a drop of acidulated solution of methyl green (0·75 per cent. of acetic acid). This is a delicate fixing agent, killing cells instantly without change of form. Complete the fixation by exposing the preparation for a quarter of an hour to vapour of osmium, and add a drop of solution of Ripart and Petit and a cover.

Or you may fix the preparation, after teasing, with vapour of osmium for half a minute to two minutes, then add a drop of methyl green, and after five minutes wash out with 1 per cent. acetic acid, and add solution of Ripart and Petit and cover.

Or you may kill and fix the cells by teasing in solution of Ripart and Petit (to which you may add a trace of osmium if you like), and afterwards stain with methyl green.

I have found Pictet's chloride of manganese (§ 386) very useful as an examination medium. A little solution of dahlia may be added to it.

HENKING'S mixture, which has been given above (§ 639), may also be found useful.

For FLEMMING'S methods for the study of the division of the ova of Echinodermata, see *Arch. f. mik. Anat.*, xx, 1881, p. 3, or previous editions of this work.

Other fixing agents, such as picric acid or weak sublimate solution, may of course be used, and in some cases doubtless should be preferred. Other stains, too, such as Bismarck brown, may be used as occasion dictates; and of course other examination media than solution of Ripart may be employed. But, for general purposes, the methyl-green-osmium-and-Ripart's-medium method gives such good results, and is so very convenient, that it may well be called the classical method for the study of fresh cells. I think great credit is due to CARNOY for his frequent insistence on the excellence and handiness of this method.

656. Some Microchemical Reactions.—*Methyl green* is a test for chromatin, in so far as it colours nothing but the chromatin *in the nucleus*. It is, however, not a perfect test, for the intensity of the coloration it produces varies greatly in different nuclei, and may in certain nuclei be extremely weak, or (apparently) even altogether wanting. In these cases other tests must be applied in order to establish with certainty the presence or absence of that element. The following suggestions are taken from CARNOY, who is, I believe, the only writer—on the zoological side, at all events—who has insisted on the necessity of applying microchemical methods in a systematic manner to the study of cells.

Chromatin is distinguished from the lecithins and from albuminoids by not being soluble, as these are, in water and in weak mineral acids, such as 0·1 per cent. hydrochloric acid. It is easily soluble in concentrated mineral acids, in alkalies, even when very dilute, and in some alkaline salts, such as carbonate of potash and biphosphate of soda. In the presence of 10 per cent. solution of sodium chloride it swells up into a gelatinous mass, or even, as frequently happens, dissolves entirely (*Biol. Cell.*, pp. 208–9). It is only partially digestible (when *in situ* in the nucleus) in the usual laboratory digestion fluids.

The solvents of chromatin that are the most useful in practice are 1 per cent. caustic potash, fuming hydrochloric acid, or cyanide of potassium, or carbonate of potash. These last generally give better results than dilute alkalies. They may be employed in solutions of 40 to 50 per cent. strength. If it be desired to remove all the chromatin from a nucleus the reaction must be prolonged, sometimes to as much as two or three days, especially if the operation be conducted on a slide and under a cover-glass, which is the safer plan.

It must be remembered that these operations must be performed on *fresh* cells, for hardening agents bring about very considerable modifications in the nature of chromatin, rendering it almost insoluble in ammonia, potash, or sodic phosphate, &c. Hydrochloric acid, however, still swells and dissolves it, though with difficulty.

Partial digestion may render service in the study of the chromatic elements of nuclei. Chromatin resists the action of digestive fluids much longer than the albumins do; so that a moderate digestion serves to free the chromosomes from any caryoplasmic granulations that may obscure them, whilst at the same time it clears up the cytoplasm.

The term "chromatin" has been used in the above paragraphs in a sense in which the term "nuclein" is employed by many writers. It is now known that there exists a whole series of nucleins, differing chiefly in respect of their richness in phosphorus and proteids. At one end of the chain is nucleic acid, with 9 to 11 per cent. of phosphorus, and without any proteids (this compound occurs in nature in the heads of spermatozoa); in the middle are what are generally termed the nucleins, consisting of proteid with varying amounts of nucleic acid; and at the other extreme are nucleins which are nearly all proteid, containing only 0·5 to 1 per cent. of phosphorus, and are in fact the same substances which have received the name of "nucleo-albumin;" they may also be termed the artificial plastins.

These substances have both been isolated from the most diverse tissues of the animal body, and have been prepared artificially. A corresponding series of nucleins exists within the nucleus itself. There are those that contain most nucleic acid; these are readily soluble in alkalies, and precipitable with difficulty by acid: *chromatin* is one of these. There are others more insoluble in alkalies and poorer in nucleic acid; these are *the plastins*, the *pyrenin* of nucleoli being one of them. And there are others even poorer in nucleic acid; these are the *nucleo-albumins* (which exist also in the cytoplasm): the paralinin, or "nuclear sap," appears to be in part composed of these, in part of phosphorus-free compounds.

There appears to be some doubt whether chromatin is or is not nucleic acid itself. The principal reactions in which it resembles nucleic acid are given by HALLIBURTON (*Goulstonian Lectures on the Chemical Physiology of the Animal Cell*, 1893, p. 574 of the Report in the *British Medical Journal*, No. 1681, March 18th, 1893, from which place also I have condensed the above remarks on the chemistry of the nucleins) as follows:—"1. It does not give Millon's nor the xantho-proteic reactions. 2. It is easily soluble in alkalies, soluble with difficulty in acids. 3. It is soluble in an acetic acid solution of potassium ferrocyanide. 4. After treatment with concentrated copper sulphate solution for twenty-four hours it loses its affinity for stains. It is not, however, dissolved by the copper sulphate as Schwarz stated. 5. It has a great affinity for anilin dyes, especially for basic dyes like methyl green. If a mixture of methyl green and acid fuchsin is employed, nucleic acid is stained green. The nucleins next richest in phosphorus are stained a blue-violet tint, whereas the phosphorus-poorest are coloured red. Now in the dividing nucleus, when the amount of chromatin is at its maximum, the nucleus stains green; whereas in the resting nucleus, where there is more pyrenin, a blue colour is observed."

It results from experiments of the nature of that mentioned under No. 5 (which, by the way, does not appear to me to be quite correctly stated), that chromatin is a *basophilous* body in EHRLICH's sense (explained in § 268), and that the albumins, and consequently cytoplasm in general are *acidophilous*.

These considerations appear to justify the employment of the term "chromatin" for the element of the nucleus that stains with methyl green, the term "nuclein" having obtained a wider extension.

See also KOSSEL, in BEHRENS, KOSSEL, und SCHIEFFERDECKER's *Das Mikroskop*, &c., ii, p. 47; the same, in *Verh. d. physiol. Ges.*, Berlin, Oct. 21st, 1892; MALFATTI, *Ber. d. naturw. med. Vereines in Innsbruck*, 1891–2; SCHWARZ, *Cohn's Beitr. z. Biol. d. Pflanzen*, v, 1, 1887, p. 1, or *Zeit. f. wiss. Mik.*, iv, 4, 1887, p. 530; ZACHARIAS, *Ber. d. deutschen botan. Ges.* x, 1893, p. 188, and xi, 1893, p. 293, or *Zeit. f. wiss. Mik.*, x, 1, 1893, p. 80, and x, 3, 1893, p. 373; LILIENFELD, *Verh. d. phys. Ges. Berlin*, 1892–3, No. 11, or *Zeit. f. wiss. Mik.*, x, 1, 1893, p. 80; ZIMMERMANN, *Zeit. f. wiss. Mik.*, xii, 4, 1896, p. 458.

For microchemical methods for the study of the distribution of assimilated iron compounds in cells, see the masterly paper of MACALLUM, in *Quart. Journ. Mic. Sci.*, No. 150, 1895, p. 175.

For the microchemical detection of phosphorus in tissue-elements see the paper of LILIENFELD and MONTI, in *Zeit. f. physiol. Chemie*, xvii, 1892,

p. 410 (Report in *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 332); also a short notice in HALLIBURTON's *Goulstonian Lectures*, 1893 (see *Brit. Med. Journ.*, March 11th, 1893, p. 505).

657. Cytological Fixing Agents.—The following is in great part taken from the numerous papers of FLEMMING in the *Arch. f. mik. Anat.* from the year 1879 onwards, and from his *Zellsubstanz Kern- und Zelltheilung*.

Osmic acid ($\frac{1}{10}$ to 2 per cent.) preserves the form of the entire cell, but swells the nuclei and rounds off nucleoli. It renders the nuclear "reticulum" undiscernible. Picric acid, either concentrated or dilute, and chromic acid, 0·1 to 0·5 per cent., are to be preferred to alcohol and other agents for the study of the cells of *Vertebrates*. Shrinking and distortion of the nuclear figures (and, with picric acid, swellings of them) are to be expected, but other agents have the same defect to a much greater degree; alcohol especially causes *entanglement* of the threads. Acetic acid does the same, and causes swelling besides. Stronger chromic acid solutions cause shrinking. Neither of these reagents is harmless as regards the nuclei of red blood-corpuscles. The salts of picric acid (potash-, soda-, and baryta-salts) are most harmful. Weak (*i. e.* not more than 1 per cent.) acetic, hydrochloric, or nitric acid, combined with clearing in glycerin and staining, may be useful for bringing out chromatin and nucleoli. Chloride of gold preserves the form well, but generally leaves the nuclear structures unstained. Nitrate of silver is hopelessly uncontrollable in its action. Alcohol has much the effect of chromic acid, but often causes a much greater shrinking of the nuclei. Bichromate of potash and chromate of ammonia bring out very sharply the appearance of a reticulum, but these appearances cannot be accepted as true (l. c., p. 334, *et seq.*).

"*Those who seek to study cell-division by means of bichromate of potash or other chromic salts are hopelessly in the wrong road.*" And this because of the injurious action of the bichromate, *not* on the body of the cell, which it preserves well, but on the chromatin structures. Chromic salts are excellent reagents for general histological work, but not for nuclear structures. They dissolve nucleoli, destroy nuclear "networks," and swell up and distort karyokinetic figures to such a degree that the appearances obtained from them are merely unnatural caricatures of the true structure.

Altmann's nitric acid method is excellent for the purpose of hunting for cell-divisions in tissues; but the minute structure of the figures is not so well preserved as it is by means of chromic or picric acid. The same must be said of Kleinenberg's picro-sulphuric acid method. (I am not alone in holding that this is a most untrustworthy cytological reagent; see, for instance, HOLL [*Sitzb. k. Akad. Wiss. Wien,* xcix, 1890, p. 311; *Zeit. f. wiss. Mik.,* ix, 1, 1892, p. 89], who found that it frequently reduced chromosomes to the state of mere lumps, "Krümeln.")

There are two fixing mixtures which may be said to be classical for cytological studies, FLEMMING'S **chromo-aceto-osmic acid** mixture, §§ 46, 47, and HERMANN'S **platino-aceto-osmic acid** mixture, § 65. As to the former of these, Flemming has the following explanations:—Attempts to omit the chromic acid from the formula did not give good results. The omission of acetic acid (as in Max Flesch's formula, § 45) causes the figures to be far less sharply brought out. The presence of acetic or formic acid in all osmium solutions is favorable to the precision of subsequent staining with hæmatoxylin, picrocarmine, or gentian violet. But mixtures of osmic and acetic acid without chromic acid (Eimer) do not give such good results as the chromo-aceto-osmic acid mixture. Mixtures of picric acid with osmic acid or with osmic and acetic acid (proportions of the latter as in the chromo-aceto-osmic mixture [§ 46], but of picric acid about 50 per cent.) fix quite as well as the chromic mixtures, but precise staining is even more difficult than with pure osmic acid preparations. Flemming concludes that the beneficial effects of the osmium in all these mixtures are to be ascribed to the instantaneous rapidity with which it kills, the function of the other acids of the mixture being to render the structures distinctly visible.

Mixtures containing osmic acid should therefore be employed whenever it is desired to fix the chromatic figures as faithfully as possible; whilst pure chromic acid should be taken whenever very sharp staining is the more important point.

For the study of achromatic figures he recommends the chromo-acetic acid mixture (§ 43), followed by staining in hæmatoxylin (anilins, he states, do not give so good results for this purpose).

For the study of polar corpuscles he recommends the osmium mixtures, or pure chromic acid followed by staining with gentian violet.

The above account stands nearly as it stood in the first edition. The state of things at present is as follows:—It is admitted by all competent observers that the chromo-aceto-osmic mixture is, with at most one or two possible exceptions, by far the best fixing agent for nuclei. But some observers have stated that it does not always preserve the cell-body well. This is a question that has been already discussed in §§ 46 and 47. I will only add here that after considerable experience I see no reason to distrust Flemming's mixture as a preservative of any kind of protoplasm, provided it be used in the proper way. It must be taken of the proper strength, it must be used with very small objects, so that it may act on all parts of them with its full strength, and not be filtered and diluted through thick walls of tissue before coming into contact with the object of study; and it must be allowed to act for the proper time.

This brings us to another point. There are *two* **Chromo-aceto-osmic** mixtures—the old *weaker* one, and the new *stronger* one. Flemming recommended the strong one primarily as affording a means of differentiating kinetic chromatin from resting chromatin. He did not recommend it as a reagent for general work. Whether of these two solutions should be used for general work? According to my experience, the strong solution *does* preserve both nuclear structures and caryoplasmic structures quite as faithfully at least as the old formula, and some structures most decidedly much better. Of course the one and the other should be taken according to the nature of the object you are dealing with; but I think it may safely be stated as a general rule that if you take the strong mixture, and fix thoroughly in it, you are not likely to go far wrong. And what is meant by a thorough fixation? Half an hour may be taken to be generally enough; but for very delicate things, such as the Nebenkern and the achromatic figure, eighteen hours or more may be desirable.

As to the **platino-aceto-osmic** mixture of Hermann, it has already been explained in § 65 that the point of superiority over Flemming's mixture that is claimed for it lies in a more faithful preservation of cytoplasm and achromatic structures.

That the alleged superiority really exists appears to be the general opinion of those who have worked with this reagent. Flemming (*Arch. f. mik. Anat.*, xxxvii, 1891, p. 685; *Zeit. f. wiss. Mik.*, viii, 3, p. 343) agrees that it gives a peculiarly sharp demonstration of spindle fibres, centrosomes, and polar corpuscles; but thinks that the chromo-aceto-osmic mixtures give a somewhat more faithful preservation of the chromatic elements. According to my experience there are slight differences in the behaviour of these two reagents which may be taken advantage of (see § 65).

Niessing (*Arch. f. mik. Anat.*, xlvi, 1895, p. 147) has the following two modifications of Hermann's mixture:

(1)	Platinic chloride, 10 per cent. solution .	25
	Osmic acid, 2 per cent.	20
	Glacial acetic acid	5
	Distilled water	50

(2) The same with saturated aqueous solution of corrosive sublimate instead of the water.

O. vom Rath's picro-platinic mixtures have been given (§ 80).

Liquid of Merkel, or the modification of it by Brass, which will be given in the chapter on Protozoa, may be found useful.

For Lindsay Johnson's platinic mixture see § 97.

Some observers have had good results with liquid of Perényi, especially for achromatic structures.

Two or three of the fixing agents proposed by other writers may also rank as first-class reagents for this kind of work. There is Rabl's **chromo-formic** acid (§ 44). Fix in this for twelve to twenty-four hours, wash out well with water, and pass into alcohol. And there is the same observer's **platinum chloride** solution (§ 64). In Rabl's paper in *Anat. Anz.*, iv, 1889, p. 21, he recommends that *Salamandra* larvæ be fixed (for twenty-four hours) in a solution of from one tenth to one eighth per cent. strength. In his earlier work he used solutions of 1 : 300 strength. Platinum chloride has the peculiarity of causing a slight shrinkage of the chromatin, which helps to bring into evidence the granules of Pfitzner and the longitudinal division of the chromosomes.

There remain to be mentioned several fixing agents with which very important work has been done. These, however, are, I think, not quite first-class reagents for the purpose, the brilliant results that have been obtained with them having been obtained rather in spite of their defects than on account of their good qualities. For instance, *acetic alcohol* is a reagent with which some of the most important work in recent cytology has been done—namely, much of that on the maturation and fecundation of the ovum of *Ascaris.* It is evident that for such an extraordinarily impenetrable object as the ovum of Ascaris, the employment of some such highly penetrating fluid as acetic acid is imperatively indicated, notwithstanding the serious defects that it may have.

Carnoy (*La Cellule,* iii, 1, 1886, p. 6) used at first a mixture of three parts of absolute alcohol with 1 of glacial acetic acid; later (*ibid.*, iii, 2, 1887) the chloroform mixture (§ 69). From five to fifteen minutes is enough for even the most resistent ova.

Van Beneden and Neyt (*Nouvelles Rech. sur la Féc. et la Division mitosique,* 1887) employed a mixture of equal parts of absolute alcohol and glacial acetic acid, or even pure acetic acid.

Acetic alcohol may be washed out with either pure alcohol, or with dilute glycerin (Calberla's formula would be a good one in many cases). For further details see *ante*, § 69.

M. Heidenhain (*Ueb. Kern u. Protoplasma,* 1892, p. 113) has been using corrosive sublimate on account of its convenience, and, above all, on account of the great facility it affords for the employment of certain stains. The beautiful figures of "attraction spheres" and other cytoplasmic structures given in this paper show that the most brilliant results may be obtained by this means. But I would remark that the figures of nuclear structures appear to me less convincing. The author figures and describes, under the name of "Lanthanin," an acidophilous caryoplasmic substance exhibiting a minutely reticular arrangement. The figures remind me of appearances which I frequently obtained in certain nuclei when working with sublimate some years ago, and which I regarded as artefacts, and in consequence was led to the abandonment of sublimate as a cytological fixative. I do not find in Heidenhain's paper that he has instituted control experiments to show that the reticular arrangement of his "lanthanin" is preformed in the nucleus, and would point out the need of such experiments before either the existence of the lanthanin reticulum or the fidelity of the reagent can be deemed established. It should further be noted that with sublimate material it is impossible to obtain with safranin, gentian violet, and some other stains, the delicate differentiations that they give with chromosmium material.

Altmann (*Arch. f. Anat. u. Entwickel.*, 1892, p. 223; *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 331) has a new fixative for resting nuclei, viz. a 2·5 per cent. solution of ammonium molybdate to which is added about 0·25 per cent. of chromic acid. He also mentions (*Die Elementarorganismen,* 1890; cf. *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 201) a fixation with nitrate or picrate of mercury, for the demonstration of his "bioblasts." I have no personal knowledge or other information concerning either of these methods.

Lemon juice (fresh, filtered) has been warmly recommended as a fixative for nuclei by van Gehuchten (*Anat. Anzeig.*, iv, 1889, p. 52). Fix for five minutes, wash well with water and stain with methyl green, and examine in liquid of Ripart and Petit.

Heat has been recommended, but I believe it to be altogether objectionable. Henking (*Zeit. f. wiss. Zool.*, xcix, 3, 1890, p. 503; *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 211) has found that it totally destroys achromatic structures.

658. Chromatin Stains.—Stains appropriate for fresh or lightly fixed tissues have been mentioned in § 655.

For sections of hardened tissues we have the choice between the finer *hæmatein* stains (or *carmalum*), and those obtained by means of *safranin*, *gentian violet*, and some other anilins, used according to the regressive method.

Of the hæmatein stains, *hæmalum* gives very good results with sublimate material; but for chrom-osmium material I decidedly prefer in general an *iron lake*, M. Heidenhain's or Benda's.

REESE LIBRARY OF THE UNIVERSITY OF CALIFORNIA

Thionin has been warmly recommended by M. Heidenhain as giving a stain even superior to that of safranin. After trial, I think the recommendation is justified. Methyl green is, in my experience, inferior for preparations destined to be mounted in balsam. Carmalum is a very precise stain, and useful for sublimate material, but is decidedly too weak for chrom-osmium material.

Babes's supersaturated safranin stain (*Arch. f. mik. Anat.*, xxii, 1883, p. 361) may also occasionally be useful. It is as follows:—A supersaturated solution of safranin in water is warmed to 60° C. and filtered warm. On cooling, it becomes turbid through the formation of small crystals. Sections are placed in a watch-glass with some of this turbid solution, and the whole is warmed for a few seconds (till the liquid becomes clear) over a spirit lamp. Allow the whole to remain for one minute, and wash out with water, and treat with alcohol and turpentine in the usual way. Tissues which do not take on the stain at once must be warmed over and over again. Clove oil must be avoided for clearing.

For some remarks of Bataillon and Koehler on the stain of borax-methylen-blue, see *Comptes Rendus*, cxvii, 1893, p. 521, or *Journ. Roy. Mic. Soc.*, 1894, p. 41.

659. Plasma Stains.—I have been unable to discover a single thoroughly satisfactory one. All of those known to me are of an imperfect electivity, in so far as it is difficult if not impossible to limit their action with the desired precision and certitude to the element that it is desired to bring into prominence by staining. Almost all of them colour too readily the enchylema or hyaloplasm at the same time as the plasmatic reticulum. And, on the other hand, there are many important elements of the cell which refuse to stain sufficiently. Spindles, for instance, can be made to stain, but only weakly at the best: it is impossible to get them to stain vigorously and at the same time distinctively.

The best, or least defective plasma stain for hardened tissues that I know of is KERNSCHWARZ; see § 334, and also my paper "Sur le Nebenkern et sur la formation du Fuseau," in *La Cellule*, xi, 2, 1896, p. 257.

After Kernschwarz, perhaps the best results I have had have been obtained by means of FLEMMING'S *orange method.* It is a very good stain for normal cytoplasmic structures, but a very poor one for the Nebenkern ("Spheres" of some authors) and other spindle relics.

BENDA'S *Safranin and Lightgrün or Säureviolett* gives sometimes splendid results, but is capricious. It is very good for the Nebenkern, less so for equatorial spindle relics.

For *Säurefuchsin* and *Orange G*, see §§ 305 and 300.

EHRLICH-BIONDI mixture is a celebrated plasma stain. It will work with chrom-osmium material. It is of no use whatever for polar corpuscles or spindle relics.

For RAWITZ' *Inversion Stain*, see § 308. I have obtained some vigorous images of polar corpuscles by this process, but I certainly do not think that it is a recommendable one.

The *Osmic Acid and Pyrogallol Process*, § 377, gives a very fair and frequently useful plasma stain; but I do not consider it to be a method of quite the first class.

The *Iron-Hæmatein Lakes* of BENDA and M. HEIDENHAIN give good plasma stains, according to the degree of extraction. The iron lake of M. HEIDENHAIN is said to be a specific stain for "centrosomes." I am not concerned to dispute the alleged fact, but must be allowed to hold that the status of the corpuscles stained by this process has not been placed on a perfectly satisfactory footing. It is said by HEIDENHAIN, and by other observers who have repeated his observations, that the reaction in question is obtained in a sharper form by combining the hæmatein stain with a foregoing stain with *Bordeaux R.* It is held by HEIDENHAIN that the foregoing stain with Bordeaux, which is a general stain taking effect on both chromatin and plasma, but having no affinity for centrosomes, so satisfies the chemical affinities of the first-named elements that they no longer hold the iron lake with the usual tenacity, but give it up freely in the extraction process, whilst the centrosomes, not having taken up so much Bordeaux, are free to hold the hæmatein strongly; which, it is stated, they do. This hypothesis is put forward as a theory under the name of *Subtractive Staining* by HEIDENHAIN, and under the name of *Tinctorial Preoccupation* by UNNA (*Zeit. f. wiss. Mik.*, xii, 4, 1896, p. 454).

The instructions given by HEIDENHAIN for the employment of the Bordeaux are not so detailed as might be desired. He states (*Arch. f. mik. Anat.*, xlii, 1894, p. 665) that the sections (sublimate sections were used by him) are to be stained for twenty-four hours or more in "a weak" solution of Bordeaux, until they have attained such an intensity of colour as that "they would just be fit for microscopic examination with high powers" (l. c., p. 440, note), and that they be then brought into the ferric alum. After mordanting and staining, the hæmatein is to be extracted in the iron alum until the chromatin has become entirely or almost entirely colourless. The Bordeaux is not supposed in this process to act as a plasma stain; it goes away in the subsequent processes. Instead of Bordeaux, "anilin blue" may be used in the same way. I have repeatedly tried the Bordeaux process, and with my objects (I have not tried Heidenhain's) have observed not the slightest difference in the preparations obtained in this way, and those obtained by the iron hæmatein without the Bordeaux. The solution of Bordeaux used by me was of 1 per cent. strength.

I have obtained some good plasma stains both with EHRLICH's *triacid* and with his *acidophilous mixture*, and for some purposes should prefer these to the Ehrlich-Biondi mixture.

HERMANN (*Arch. f. mik. Anat.*, xxxvii, 4, 1891, p. 583) recommends a modification of the hæmatoxylin impregnation method of PAL:—Testes of *Proteus* put for twelve to eighteen hours into hæmatoxylin, 1 grm.; absolute alcohol, 70 c.c.; water, 30 c.c., in the dark, and washed out for the same time in 70 per cent. alcohol, also in the dark. Sections having been made are treated with dilute pale rose-coloured solution of permanganate of potash till they turn ochre-coloured, rinsed in water, and washed out for removal of the brown peroxide of manganese in Pal's oxalic acid mixture diluted with 5 to 10 volumes of water. They are finally stained for three to five minutes in safranin.

See also on this subject the paper of HERMANN, "Methoden zum Studium des Archoplasmas und der Centrosomen tierischer und pflanzlicher Zellen," in *Ergebnisse der Anatomie*, Band ii, 1892 (1893), p. 23.

For Heidenhain's *Vanadium hæmatoxylin*, see Cohn in *Anat. Hefte*, 1895, p. 302, or *Zeit. f. wiss. Mik.*, xii, 3, 1896, p. 359. The achromatic structures of the ovum of *Ascaris* are best demonstrated, according to van Beneden et Neyt (*Nouv. Rech.*) by fixing with acetic alcohol (*ante*, § 657), and bringing the ova into one-third glycerin in which is dissolved a little malachite green. The "sphères attractives" stain green. See also Boveri's *Zellen-Studien* (in *Jen. Zeitschr. f. Naturw.*, xxi, 1887, p. 423, or sold separately). Herla (*Arch. de Biol.*, xiii, 1893, p. 423) advises a mixture of vesuvin 0·25, malachite green 0·25, distilled water 100, and glycerin 10; the stain to be washed out with dilute glycerin.

660. Nucleus of Balbiani ("Noyau Vitellin," "Cellule Embryogène") (*Zool. Anz.*, 1883, p. 659).—This may be observed in the fresh state, without the addition of any reagent, in the ova of some animals, amongst others a great number of Arachnida and Myriapoda. It may be brought out more distinctly by treating the ova with a mixture of equal parts of acetic acid and 1 per cent. osmic acid, to which is added a little sodium chloride. This mixture does not render ova so granular as pure dilute acetic acid. For sections, stain by Henneguy's permanganate safranin process.

661. Cell-granules.—These for the most part undoubtedly metabolic products are best studied in gland-cells and blood- and lymph-corpuscles, and in certain elements belonging to the group of connective tissues, and the reader is therefore referred to the sections on "Connective Tissues" for the appropriate methods. It will suffice here to state that the most generally employed stains for cell-granules are the mixtures of Ehrlich.

For Altmann's "Bioblasts," see, besides that author's *Studien über die Zelle*, 1886, his *Die Elementarorganismen*, Leipzig, 1890, and a paper in *Arch. f. Anat. u. Entwickel.*, 1892, p. 223; also *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 199; ix, 3, 1893, p. 331; and L. and R. Zoja, in *Mem. R. Ist. Lombardo di Sci. e Lettere*, xvi, 3, vii, p. 237. Omitting minutiæ and variations, it may be said that these granules may be demonstrated by fixing for twenty-four hours in a mixture of equal parts of 5 per cent. bichromate of potash and 2 per cent osmic acid, imbedding in paraffin, staining sections for a minute on the slide held over a flame with a solution of 20 grms. of acid fuchsin in 100 c.c. of anilin water (p. 190), and washing out with saturated alcoholic solution of picric acid diluted with 2 volumes of water, heat being used as before to aid the differentiation, and finally clearing with xylol and mounting in balsam. FISCHER (*Anat. Anz.*, ix, 1894, No. 22, p. 678) has given reasons for believing that these granules may consist, in part at all events, of precipitates of albuminates (principally peptones) thrown down by the fixing agents (see also § 657).

662. More Special Methods.—For accounts of the methods that have been employed in the study of tissue-cells, and more especially of sexual products, now suppressed for want of space, see previous editions, or the *Traité des Méthodes techniques de l'Anat. microscopique*, LEE *et* HENNEGUY.

CHAPTER XXIX.

TEGUMENTARY ORGANS.

663. Epithelium.—One of the chief methods of obtaining preparations giving instructive surface views of epithelia is the *nitrate of silver* method. For this see *ante*, § 358, *et seq.*, in the chapter on "Impregnation Methods." The reader may also consult with advantage the admirable instructions given by Ranvier in his *Traité technique*, p. 246, *et seq.*, and the memoir of Tourneux and Hermann in the *Journ. de l'Anat.*, 1876, p. 200.

The *perchloride of iron and pyrogallic acid* method of the Hoggans, § 379, and the *osmic acid and pyrogallic acid* process, § 377, may also be found useful here. But in many cases impregnation with *methylen blue* will doubtless be found preferable.

Sections are easily made by the usual methods. The best hardening agent for *skin* appears to be Müller's solution. This was the conclusion of F. E. Schultze in 1867 (*Arch. f. mik. Anat.*, p. 145); and it is that of Tizzoni, the author of important researches on this organ (*Bull. delle Sc. med. di Bologna*, 1884, p. 259), and of Behn (*Arch. f. mik. Anat.*, xxxix, 1892, p. 581). Simple bichromate of potash solution will do about as well.

For *glandular epithelium* it is frequently better to employ a chromic acid liquid, or osmic acid (see, for example, Ranvier, loc. cit., p. 258, *et seq.*), or absolute alcohol (Blaue, *Arch. f. Anat. u. Phys.*, 1884, p. 231); "Kleinenberg" is not so good.

Macerating Media.—For soft epithelia, mild macerating agents, such as iodised serum, one-third alcohol, saliva, or Schultze's mixture of saliva and solution of Müller, or a mixture of saliva with three to four volumes of physiological salt solution (Bizzozero, *Intern. Monatsschr. f. Anat.*, 1885, p. 278);—for hard epithelia, energetic dissociating agents, such as 40 per cent. solution of caustic potash.

Minot (*Amer. Natural.*, xx, 1886, p. 575; cf. *Journ. Roy. Mic. Soc.*, 1886, p. 872) recommends maceration for several days in 0·6 per cent. solution of sodium chloride containing 0·1 per cent. of thymol, which allows the isolation of the epidermis of embryos, and is useful for the study of the development of hairs.

Another method, given by Mitrophanow (see *Zeit. f. wiss. Mik.*, v, 4, 1888, p. 513), is as follows:—An embryo of axolotl is fixed for a quarter of an hour in 3 per cent. nitric acid, and then brought into one-third alcohol. After an hour the epidermis begins to come away in places; and if the embryo be put for twenty-four hours into stronger spirit, it will come away almost entirely.

For the purpose of separating the epidermis from the corium, Loewy (*Arch. f. mik. Anat.*, xxxvii, 1891, p. 159; *Zeit. f. wiss. Mik.*, viii, 2, 1891, p. 222) recommends macerating for twenty-four to forty-eight hours, at a temperature of about 40° C., in 6 per cent. pyroligneous acid. Acetic acid of $\frac{1}{3}$ per cent. (Philippson) is also good.

For *ciliated epithelium*, see the methods of Englemann under "Mollusca."

664. Prickle-Cells and Intercellular Canals.—Besides maceration, which is one of the most important of the methods for the study of these objects, impregnation may be useful. Mitrophanow (*Zeit. f. wiss. Zool.*, 1884, p. 302, and *Arch. f. Anat. u. Phys.*, 1884, p. 191) recommends the following process:—Wash with distilled water the tail of an axolotl larva; put it for an hour into 0·25 per cent. solution of gold chloride with one drop of hydrochloric acid to a watch-glassful of the solution; wash, and reduce in a mixture of one part of formic acid with six parts of water.

On this subject see the important memoirs of Ide, in *La Cellule*, iv, 2, 1888, p. 409, and v, 2, 1889, p. 321.

665. Plasma-fibrils of Epithelium.—Kromayer's process for demonstrating his intra- and intercellular fibrils of epithelia (*Arch. f. mik. Anat.*, xxxix, 1892, p. 141; *Zeit. f. wiss. Mik.*, ix, 1, 1892, p. 84, and ix, 3, 1893, p. 355) is as follows:—Sections are stained for five minutes in a mixture of equal volumes of anilin water (p. 190) and concentrated aqueous solution of methyl violet 6 B. They are well washed in water and treated with solution of iodine in iodide of potassium until they become blue-black (one to thirty seconds). They are again washed with water, dried with blotting-paper, and treated with a mixture of 1 vol. of anilin to 2 vols. of xylol until sufficiently differentiated, when they are brought into pure xylol. Very thin sections will require more xylol in proportion to the anilin, viz. 1 : 3 or 1 : 4; thicker ones may require more anilin, viz. 3 : 5 or 3 : 3. Gentian or Krystallviolett will do instead of methyl violet, but not quite so well. For a discussion of the points of difference between the fibrils of Kromayer and the fibres of HERXHEIMER, see EHRMANN, and JADASSOHN, *Arch. f. Dermatol. u. Syphilis*, 1892, 1, p. 303; *Zeit. f. wiss Mik.*, ix, 1893, p. 356.

For the same object UNNA (*Monatsh. f. prakt. Dermatol.*, xix, 1894, p. 1 and p. 277, *et seq.*; *Zeit. f. wiss. Mik.*, xii, 1, 1895, pp. 61, 63) has given a whole series of minutely detailed methods, from which the following are some extracts:

1. WASSERBLAU-ORCEIN.—Stain sections for ten minutes in a neutral aqueous 1 per cent. solution of Wasserblau, rinse with water and stain for five or ten minutes in a neutral alcoholic 1 per cent. solution of Grübler's orcein. Dehydrate, clear, and mount in balsam. This method may be varied as follows:

(*a*) Ten minutes in the Wasserblau, and thirty minutes or more in the orcein.

(*b*) Take for the second stain an *acid* solution of orcein.

(*c*) Stain for only one minute in the Wasserblau, but for thirty or more in the neutral orcein.

2. Stain for half an hour or more in a strong solution of hæmalum, rinse with water, stain for half a minute in a saturated aqueous solution of picric acid, and dehydrate for thirty seconds in alcohol containing 0·5 per cent. of picric acid.

3. Hæmalum for two hours, neutral orcein as above for ten to twenty minutes.

Besides these methods, UNNA gives half a dozen others which can hardly be usefully detached from the theoretical part of his papers.

666. Horn, Hair, and Nails.—The elements of hairs and nails may be isolated by prolonged maceration in 40 per cent. potash solution, or by heating with concentrated sulphuric acid. See also von Nathusius, *Zool. Anz.*, xv, 1892, p. 395.

Horny tissues stain well in safranin or gentian violet (Reinke, *Arch. f. mik. Anat.*, xxx, 1887, p. 183; *Zeit. f. wiss. Mik.*, iv, 3, 1887, p. 383).

667. Tactile Hairs.—Ranvier (*Traité*, p. 914) recommends for the study of the nerve-endings the boiled formic acid and gold-chloride method, § 371. A tactile hair having been isolated with its bulb, and its capsule incised, is put for about an hour into the formic acid and gold-chloride mixture, the gold is reduced in slightly acidulated water, hardening is completed in alcohol, and longitudinal and transverse sections are made. For Drasch's method see *Zeit. f. wiss. Mik.*, iv, 4, 1887, p. 492.

668. Skin Nerves.—Wolters (*Arch. f. Dermatol. u. Syphilis*, 1892; *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 360) employs the chloride of vanadium and hæmatoxylin method, which will be given in the chapter on "Nerve-cell and Cell-process Stains."

669. Intra-epidermic Nerve-fibres.—May be studied by the gold method. Ranvier (*Traité*, p. 900) recommends the boiled formic acid and gold-chloride method, § 371.

He also (p. 910) recommends this method for the study of the tactile menisci of the pig's or mole's snout.

Pieces of skin are impregnated as directed § 371, and after reduction are brought into alcohol, which completes the hardening, and stays the further reduction of the gold. Sections are then made.

For the study of the tactile menisci of the snout, Ranvier also recommends the lemon juice and gold-chloride method, § 372.

The methylen blue impregnation method should also be employed in the study of these nerve-endings.

670. Tactile Corpuscles (Fischer, *Arch. f. mik. Anat.*, 1875, p. 366).—Fischer employed the gold method of Löwit—see § 370. Ranvier (*Traité*, p. 918) also recommends this method, as well as his two gold methods, §§ 371, 372. Pieces of skin are first impregnated whole, then hardened by alcohol and sectioned. He finds (as do other authors) that osmic acid and picro-carmine are invaluable aids to the study of these structures, and to that of the corpuscles of Pacini. Purpurin and hæmatoxylin may also be used for after-staining. See RANVIER, *Traité*, p. 919; and see also LANGERHANS, *Arch. f. mik. Anat.*, 1873, p. 730; KULTSCHIZKY, *ibid.*, 1884, p. 358; and SMIRNOW, *Intern. Monatschr. f. Anat.*, &c., x, 1893, 6, p. 241; *Zeit. f. wiss. Mik.*, x, 2, 1893, p. 254 (this observer recommends, besides the gold method of Löwit, the rapid bichromate of silver method of GOLGI).

671. Corpuscles of Herbst and Corpuscles of Grandry.—DOGIEL (*Arch. f. Anat. u. Entwickel.*, 1891, p. 182; *Zeit. f. wiss. Mik.*, viii, 4, 1892, p. 520) prefers the methylen blue method (Chap. XVII). Four per cent. solution of methylen blue, warmed to 40° C., is injected into blood-vessels of the heads of ducks or geese; pieces of skin are removed from the beaks, sectioned in pith, and the sections brought on to slides and moistened with aqueous or vitreous humour from the animal, and left for a few minutes exposed to the air (it is well to add to the aqueous or vitreous humour a few drops of $\frac{1}{16}$ per cent. methyl blue solution). After about ten to thirty minutes the nerve-endings are seen to be stained, and the sections are then brought into picrate of ammonia, and treated as described in the chapter on "Methylen Blue." GEBERG (*ibid.*, x, 2, 1893, p. 244) has also employed this method. He has also made use of simple osmic acid, and of the gold method of ARNSTEIN, which is as follows:

Pieces of skin are macerated for twenty-four hours in lime water, after which the horny layer may easily be removed. This being done, the skin is cut up into small pieces which are put for five minutes into a 0·25 per cent. solution of chloride of gold. Reduction sets in very rapidly, and the preparations become brown in a few minutes. The reduction is completed by putting them for twenty-four hours into distilled water. During this time there forms a granular precipitate which is removed by putting the pieces into a 0·25 per cent. solution of cyanide of potassium and brushing them vigorously with a camel's-hair brush; after which they are mounted in damar. Geberg used the gold chloride of 0·5 per cent. strength, and allowed it to act for thirty minutes.

See also the method of Carrière, *Arch. f. mik. Anat.*, 1882, p. 146, or previous editions of this work.

672. Corpuscles of Meissner and of Krause (Cornea and Conjunctiva Bulbi and Palpebrarum) (Dogiel, *Arch. f. mik. Anat.*, xxxvii, 1891, p. 602, and xliv, i, 1894, p. 15).—A fresh bulb should be enucleated *in toto,* and incised along a line running parallel to the equator and 5 to 8 mm. behind the corneal margin. The anterior segment thus obtained is freed from the ciliary body, lens, &c., the conjunctiva being left *in situ* on the cornea. It is then cut into pieces which are stained for an hour to an hour and a half in methylen blue + aqueous humour, and further treated for examination and preservation as described in the chapter on "Methylen Blue."

See also Longworth's methods, *Arch. f. mik. Anat.*, 1875, p. 655.

673. Similar Objects.—Papillæ Foliatæ of the Rabbit (Hermann).—See *Zeit. f. wiss. Mik.*, v, 4, 1888, p. 524. **Olfactive Organs of Vertebrates** (Dogiel, *Arch. f. mik. Anat.*, 1887, p. 74). **Organs of a "Sixth Sense" in Amphibia** (Mitrophanow).—See *Zeit. f. wiss. Mik.*, v, 4, 1888, p. 513. (This paper contains some details as to staining with "Wasserblau," for which see also *Biol. Centralb.*, vii, 1887, p. 175.) **Nerve-endings in Tongue of Frog** (Fajerstain [Feuerstein], *Arch. de Zool. expér. et gén.*, vii, 1889, p. 705; *Zeit. f. wiss. Mik.*, vii, 3, 1889, p. 357).—(Amongst other methods for the study of the terminal discs, the methylen blue method is recommended; see previous editions). **Tongue of Rabbit**, von Lenhossék, *Zeit. f. wiss. Mik.*, xi, 3, 1894, p. 377 (Ramón y Cajal's double Golgi-method).

674. Cornea.—There are three chief methods for the study of the corneal tissue—the methylen blue method, the silver method, and the gold method.

For the *methylen blue method* see Chap. XVII, particularly §§ 295 and 296.

Negative images of the corneal cells are easily obtained by the dry *silver method* (Klein). The conjunctival epithelium should be removed by brushing from a living cornea, and the corneal surface well rubbed with a piece of lunar caustic. After half an hour the cornea may be detached and examined in distilled water.

In order to obtain *positive* images of the fixed cells the simplest plan (Ranvier) is to macerate a cornea that has been prepared as above for two or three days in distilled water. There takes place a secondary impregnation, by which the cells are brought out with admirable precision.

The same result may be obtained by cauterising the cornea of a living animal as above, but allowing it to remain on the living animal for two or three days before dissecting it out, or by treating a negatively impregnated cornea with weak salt solution or weak solution of hydrochloric acid (His).

But the best positive images are those furnished by *gold chloride*. Ranvier prefers his lemon-juice method to all others for this purpose (see § 372). It is important that the cornea should *not remain too long in the gold solution*, or the nerves alone will be well impregnated.

Ranvier also recommends this method as being the best for the study of the nerves.

Rollett (Stricker's *Handbuch*, p. 1115) recommends a double impregnation with silver followed by gold for obtaining gold-stained *negative* images. A cornea having been treated *for a short time only* with 0·5 per cent. silver nitrate solution, and the silver reduced, is treated with 0·5 per cent. gold chloride solution. The brown stain of the silver disappears immediately the preparation is placed in the gold solution; after a few minutes the preparation is exposed to the light in acidulated water. Reduction of the gold rapidly takes place, and in the place of the former brown stain of the silver the ground-substance shows the well-known blue of reduced gold. The cells are, however, visible, being recognisable by their granular appearance and pale yellow tint.

Renaut (*Comptes Rend.*, 1880, 1er sem., p. 137) gives the following process for corneal corpuscles:—Cornea of frog: formic acid, 20 per cent., ten minutes; gold chloride, 1 per cent., twenty-four hours; formic acid, 33·3 per cent., twenty-four hours.

Hoyer's method has been given, § 374.

675. Cornea, other Methods (Rollett, Stricker's *Handb.*, p. 1102).—Rollett strongly recommends the following plan:—A fresh cornea is placed (in humor aqueus) in a moist chamber, and exposed to the action of iodine vapour. As soon as it has become brown the epithelium may easily be peeled off. If the reaction is not complete the cornea may be put back into the iodine chamber. When sufficient iodine has been absorbed the preparation may be examined, and it will be found that the network of corneal cells is brought out with an evidence hardly inferior to that of gold preparations. The method never fails, which is not the case with the gold method. It is admirable as a fixing method.

For dissociation of the fibres Rollett recommends maceration in a solution of permanganate of potash or a mixture of this solution with alum. As soon as the tissue has become brown it is shaken in a test-tube with water, and breaks up into fibres and bundles of fibres.

TARTUFERI (*Anat. Anz.*, v, 1890, p. 524; *Zeit. f. wiss. Mik.*, vii, 3, 1890, p. 365) has the following method for demonstrating corneal cells and the ramifications of their processes:—A cornea is placed for three or more days in a solution of 15 grammes of hyposulphite of soda to 100 c.c. of distilled water, kept at a temperature of about 26°, then removed for two days into water containing very finely powdered chloride of silver (if left longer the corneal cells will not be stained, but innumerable elastic fibres will be demonstrated). The preparations are said to be permanent. Some further details are mentioned in *Zeit. f. wiss. Mik.*, xi, 3, 1894, p. 346.

676. Crystalline (Hardening of) (LÖWE, *Arch. f. mik. Anat.*, 1878, p. 557).—A fresh bulb is placed in a vessel containing *several litres* of 1 per cent. bichromate of potash solution, which is frequently changed for stronger solutions until the strength of a cold saturated solution is attained. The bulb must remain in this for at least *a year and a half*, in order that the crystalline may attain the right degree of hardness. For **Maceration**, use Max Schultze's sulphuric acid solution, *supra*, § 557.

Formaldehyde may perhaps be found useful for hardening.

CHAPTER XXX.

MUSCLE AND TENDON (NERVE-ENDINGS).

Striated Muscle.

677. Muscle-cells.—For the study of these and allied subjects see, *inter alia*, BEHRENS, KOSSEL, und SCHIEFFERDECKER, *Das Mikroskop*, &c., vol. ii, pp. 154—161; also, for the application of the gold method to the study of muscle-cells, SCHÄFER, *Proc. Roy. Soc.*, xlix, 1891, p. 280; or *Journ. Roy. Mic. Soc.*, 1891, p. 683.

678. Sarcolemma.—Besides the places quoted in last section, see SOLGER, *Zeit. f. wiss. Mik.*, vi, 2, 1889, p. 189 (small pieces of fresh muscle teased and examined in cold saturated solution of carbonate of ammonia).

679. Sections (ROLLETT, *Denkschr. math. naturw. Kl. k. Akad. Wiss. Wien*, 1885; *Zeit. f. wiss. Mik.*, 1886, p. 92).—Besides the usual section methods, the following methods of Rollett should be noted:—(1) The method mentioned (§ 183) of freezing living tissue in white of egg. (2) The same method applied to recently fixed muscle.

680. Dissociation.—See Chap. XXV.

LANGERHANS' methods for *Amphioxus* (*Arch. f. mik. Anat.*, 1875, p. 291).—For isolation of the muscle-plates macerate the fresh animal in 20 per cent. nitric acid.

For isolation of the nervous system macerate an animal for three days in 20 per cent. nitric acid, then place it for twenty-four hours in water, and shake forcibly. The *whole* of the nervous system may thus be separated, almost down to the finest peripheral terminations of nerves.

681. Nerve-endings.—For the study of nerve-endings in muscle, both motor and sensory, the four chief methods are the methylen-blue method, the gold method, the silver method, and the bichromate of silver method of Golgi.

682. Nerve-endings—the Methylen-blue Method.—The principles of the impregnation of nerve-tissue with methylen blue have been explained in Chap. XVII.

Biedermann's procedure for the muscles of *Astacus* has been indicated in § 291 (see also *Zeit. f. wiss. Mik.*, vi, 1, 1889, p. 65). After impregnating as there directed the carapace should be opened, and the muscles exposed to the air in a roomy moist chamber for from two to six hours, in order that the stain may differentiate. The abdominal and caudal muscles are those which give the best results.

For *Hydrophilus piceus*, Biedermann proceeded by injecting 0·5 c.c. of methylen-blue solution between the ultimate and penultimate abdominal rings, in the ventral furrow, and keeping the animals alive in water for three to four hours. After this time the thorax should be opened by two lateral incisions, and the muscles of the first pair of legs (which are the most suitable) removed and exposed to the air for three or four hours in a moist chamber, and finally examined in salt solution.

Gerlach (*Sitzb. k. math.-phys. Cl. k. bayer. Akad. Wiss. München*, 1889, ii, p. 125; *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 220) injected frogs, either through the abdominal vein or through the aorta, with 4 to 5 c.c. of a 1 : 400 solution in 1 per cent. salt solution, and examined pieces of muscle (preferably the head and eye muscles) in serum of the animal, afterwards fixing the preparations with picrate of ammonia and mounting in glycerin jelly.

The procedure of Dogiel has been given in § 291.

683. Nerve-endings—the Gold Method.—Fischer (*Arch. f. mik. Anat.*, 1876, p. 365) used the gold method proposed by Löwit (*Wien. Sitzgsber.*, Bd. lxxi, Abth. 3, 1875, p. 1), and employed by himself in his researches on the tactile corpuscles (*Arch. f. mik. Anat.*, xii, p. 366). See *ante*, § 370.

Biedermann, in the paper quoted in the last section, recommends for *Astacus* a similar procedure, the preliminary treatment with formic acid being omitted, and the muscles being put for a couple of days into glycerin after reduction in the acid.

The procedure of Trinchese (*Mem. R. Accad. Ist. Bologna*, 5, ii, p. 279; *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 238) is also practically identical.

Ranvier (*Traité*, p. 813) finds that for the study of the motor terminations of Batrachia the best method is his lemon-juice and gold-chloride process (§ 372). The delicate elements of the arborescence of Kühne are better preserved by this method than by the simple method of Löwit.

For the study of the motor plates of reptiles, fishes, birds, and mammals, he finds (*ibid.*, p. 826) that his formic acid and gold-chloride method (§ 371) gives preparations infinitely superior to those obtainable by the method of Löwit; but the lemon-juice method is still better, especially for lizards and mammals. The branches of the terminal arborescence are more regular than in preparations obtained by the formic acid process.

684. Nerve-endings—the Silver Method.—Ranvier finds that the silver nitrate method of Cohnheim is also useful. He employs it as follows (*ibid.*, p. 810):—Portions of muscle (gastrocnemius of frog) having been very carefully teased out in fresh serum are treated for ten to twenty seconds with nitrate of silver solution of 2 to 3 per 1000, and exposed to bright light (direct sunlight is best) in distilled water. As soon as they have become black or brown they are brought into 1 per cent. acetic acid, where they remain until they have swelled up to their normal dimensions (the swelling induced by the acid serving to make up for the shrinkage caused by the nitrate of silver). They are then examined in a mixture of equal parts of glycerin and water.

This process gives *negative* images, the muscular substance is stained brown, except in the parts where it is protected by the nervous arborescence, which itself remains unstained. The gold process gives *positive* images, the nervous structures being stained dark violet.

685. Nerve-endings—the Bichromate of Silver Method.—The osmium bichromate and silver method of Golgi has been successfully applied by Ramón y Cajal to the study of the terminations of nerves and of tracheæ in the muscles of insects, and is doubtless susceptible of still wider applications. The process used by him is the rapid one.

In *Zeit. f. wiss. Mik.*, vii, 3, 1890, p. 332, he gives it as follows:—Fresh *muscle of insects* put into a mixture of 20 parts 3 per cent. bichromate solution and 5 parts 1 per cent. osmium solution for twelve to twenty-four hours, then into 0·75 per cent. nitrate of silver for one day, then alcohol of 40 degrees (presumably Baumé, *i. e.* about 90 per cent.), then clove oil followed by resinified turpentine and (if I understand rightly) balsam.

686. Nerve-endings—other Methods.—For the following see previous editions :—BREMER (*Arch. f. mik. Anat.*, 1882, p. 195); CIACCIO (*Journ. de Micrographie*, 1883, p. 38); WOLFF (*Arch. f. mik. Anat.*, 1881, p. 355); CARL SACHS (*ibid.*); KRAUSE (*Intern. Monatschr. f. Anat. u. Hist.*; *Zeit. f. wiss. Mik.*, 1885, p. 547); NEGRO (*Zeit. f. wiss. Mik.*, v, 2, 1888, p. 240); SIHLER, *Verhand. d. Physiol. Ges. Berlin*, 1894–5; *Zeit. f. wiss. Mik.*, xii, 3, 1896, p. 389 (these two are hæmatoxylin stains); BOCCARDI (*Zeit. f. wiss. Mik.*, iv, 4, 1887, p. 492); KÜHNE (*Zeit. f. Biol.*, xxiii, v, 1887, p. 1; *Zeit. f. wiss. Mik.*, iv, 4, 1887, p. 495—this paper contains a critical review of the different gold methods); GOLGI (*Mem. delle R. Accad. di Sci. di Torino*, ii, 32); MARSHALL (*Quart. Journ. Mic. Sci.*, 1890, p. 73; *Journ. Roy. Mic. Soc.*, 1890, p. 404); MAYS (*Zeit. f. Biol.*, 1884, p. 449; *Zeit. f. wiss. Mik.*, 1885, p. 242).

Tendon.

687. Corpuscles of Golgi (RANVIER, *Traité*, p. 929).—Take the tendon of the anterior and superior insertion of the gemini muscles of the rabbit. Free it as far as possible from adherent muscle-fibres. Treat it according to the formic acid and gold method (§ 371), and after reduction of the gold scrape the tendon with a fine scalpel, in order to remove the muscle-fibres that mask the "musculo-tendinous organs."

688. Corpuscles of Golgi (in the tendons of the motores bulbi oculi) (VON MARCHI's methods, *Archivio per le Scienze Mediche*, vol. v, No. 15).—The enucleated eyes, together with their muscles, were put for not less than three days into 2 per cent. bichromate of potash. The muscles and tendons were then carefully dissected out, stained with gold chloride and osmic acid (Golgi's method, *supra*, § 686), and by the methods of MANFREDI, given in § 374.

Mount all these preparations in glycerin (balsam clears too greatly). The methods only succeed completely during fine sunny weather.

689. Corpuscles of Golgi (CATTANEO, *Arch. ital. de Biol.*, x, 1888, p. 337).—The method here recommended is the arsenic acid gold method of Golgi quoted above, § 686.

See also Ruffini (*Atti R. Acc. Lincei Roma,* 1892, p. 442; *Zeit. f. wiss. Mik.,* ix, 2, 1892, p. 237), who recommends the method of Fischer.

690. Corpuscles of Golgi (Ciaccio, *Mem. R. Acc. Sci. Bologna* [4], t. x, 1890, p. 301; *Zeit. f. wiss. Mik.,* vii, 4, 1891, p. 507). —For Amphibia the usual gold methods are not satisfactory, because the ground-substance of the tendon takes the stain at the same time as the nerve-endings. Pieces of tendon should be put into 0·1 per cent. hydrochloric acid or 0·2 per cent. acetic acid until quite transparent. They should then be put for five minutes into a mixture of 0·1 per cent. gold chloride and 0·1 per cent. potassium chloride. After that they are put back into the acetic acid, and remain there for a day in the dark, and for two or three hours more in the sunlight. When they have become somewhat violet they are put for a day into 0·1 per cent. osmic acid, and finally mounted in Price's glycerin acidulated with 0·5 per cent. of acetic or formic acid.

691. The Methylen-blue Method.—I find no mention of the application of this method to the study of the relations of nerve and tendon, for which it would seem *a priori* to be peculiarly suitable.

Smooth Muscle.

692. Test for Smooth Muscle (Retterer, *Comptes Rend. Soc. Biol.,* iv, 1887, p. 645; *Journ. Roy. Mic. Soc.,* 1888, p. 843).— If a specimen of tissue be fixed in a mixture of ten volumes of 90 per cent. alcohol and one volume of formic acid, well washed, and stained for twenty-four to thirty-six hours with alum-carmine, the cytoplasm of smooth muscle will be found to be stained red, whilst connective-tissue cells remain unstained, and are swollen.

693. Smooth Muscle—Isolation of Fibres (Schwalbe, *Arch. f. mik. Anat.,* 1868, p. 394).—Maceration in weak chromic acid solution (0·02 per cent. proved a generally useful strength). This is a better reagent than osmic acid, 1 per cent. acetic acid (Moleschott), weak sulphuric acid, pyroligneous acid (Meissner), 20 per cent. nitric acid (Reichert), 32 to 35 per cent. potash solution (Moleschott), as it preserves better than any of these the finer structure of the cells.

GAGE's methods.—See *Journ. Roy. Mic. Soc.*, 1887, p. 327; and §§ 539, 551, and 555, *ante.*

MOBIUS, liquid for maceration of the muscle of *Cardium* (see above, § 550).

BALLOWITZ, muscle of Cephalopoda, see *Arch. f. mik. Anat.*, xxxix, 1892, p. 291; *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 344.

694. Smooth Muscle, Specific Stain for (UNNA, *Monatsh. f. prakt. Dermatol.*, xix, 1894, p. 533; *Zeit. f. wiss. Mik.*, xii, 2, 1895, p. 243).—Sections stained for ten minutes in polychromatic methylen blue solution, rinsed in water, and brought for ten minutes into 1 per cent. solution of red prussiate of potash. This fixes the colour, so that the sections will now bear differentiating with acid alcohol. They are treated accordingly with alcohol acidified with 1 per cent. of hydrochloric acid for about ten minutes (until the collagen ground comes out white). Absolute alcohol, essence, balsam.

In the same place see also another stain with acid orcein, hæmatein, Säurefuchsin, and picric acid.

695. Iris (DOGIEL, *Arch. f. mik. Anat.*, 1886, p. 403).—An enucleated eye is divided into halves, and the anterior one with the iris brought for some days into a mixture of two parts one-third alcohol and one part 0·5 per cent. acetic acid. The iris can then be isolated, and split from the edge into an anterior and posterior plate, and these stained according to the usual methods.

696. Iris (KOGANEI, *Arch. f. mik. Anat.*, 1885, p. 1).—The pigmented epithelium can be removed by brushing with a small brush after prolonged maceration in solution of Müller. The pigment may also be bleached by chlorine water, which, however, should only be allowed to act for a few hours, until the pigment has become of a light brown; complete decoloration may be obtained by prolonging the reaction for twenty-four hours, but then the tissues suffer. (See *Journ. Roy. Mic. Soc.*, 1886, p. 874.)

See also CANFIELD, in *Arch. f. mik. Anat.*, 1886, p. 121; and DOSTOIEWSKY, *ibid.*, p. 91 (sections stained with hæmatoxylin and eosin, or [List, *Zeit. f. wiss. Mik.*, iii, 4, 1886, p. 514] with Renaut's hæmatoxylic glycerin).

697. Stomach of Triton (see Stilling and Pfitzner, in *Arch. mik. Anat.*, 1886, p. 396).

698. Bladder of Frog, Innervation of (Wolff, *Arch. f. mik. Anat.*, 1881, p. 362).—A frog is killed and a solution of gold chloride of 1 : 20,000 injected into the bladder through the anus. (If the injection flows out on removal of the syringe, tie the frog's thighs together.) Now open the frog, dissect away the attachments of the bladder, ligature the intestine above the bladder, and cut away the abdomen of the frog so as to have in one piece bladder, rectum, and hind legs. (All this time the bladder must be kept moist with weak gold solution.) The bladder and the rest are now put into gold solution of 1 : 2000 for four hours; the bladder is then excised, slit open, and pinned (with hedgehog spines) on to a cork (outside downwards). Place it under running water until all the epithelium is washed away. Use a pencil if necessary. Put for twenty-four hours into gold solution of 1 : 6000. Wash in pure water, and put away in the dark "for some time" in acidulated water, and finally reduce in fresh water in common daylight. The muscles should be pale blue-red; medullated nerves dark blue-red; sympathetic nerves and ganglia carmine-red. Ranvier (*Traité*, p. 854) recommends one or the other of his two gold processes. The bladder of frogs should be carefully distended by injection of the lemon juice or gold chloride and formic acid through the cloaca.

See also the method followed by Bernheim, *Arch. f. Anat. u. Physiol.*, Physiol. Abth., 1892, supp., p. 29; *Zeit. f. wiss. Mik.*, x. 4, 1893, p. 484; likewise a gold method.

The *methylen-blue* method would appear to be very much indicated for this object, but has not been used for it so far as I am aware.

CHAPTER XXXI.

NEUROLOGICAL METHODS—INTRODUCTION AND SECTION METHODS.

699. Introduction.—The technique of the microscopic anatomy of the nervous system is highly special. The ordinary methods of microscopic anatomy are not sufficient to elucidate either the delicate and complicated structure of nervous elements, or their anatomical relations. These problems can only be successfully attacked by means of special methods of hardening, and the employment of highly specific methods of coloration.

Histological research into the structure of the nervous system pursues two ends. Either it is desired to elucidate the minute structure of the nervous elements or neurons (neurites—FISH), that is to say, the internal organisation of nerve-cells and nerve-fibres: the processes employed to this end forming a group of *cytological* methods. Or it is desired to study the form of nerve-cells, the exact distribution of the divers groups of nerve-cells in the grey matter, the connections that are formed by means of nerve-fibres between these groups of nerve-cells or "nuclei," and to follow out the intricate course of the tracts of fibres that enter into the constitution of the white matter of the cerebro-spinal axis. The processes employed in all these researches form a group of the *anatomical* methods of neurology. It is more especially in this group that we find highly special methods of selective coloration. This group may be divided as follows.

A. *Nerve-fibres.*

(*a*) Myelin stains; comprising the methods of WEIGERT, and similar methods.

(*b*) Axis-cylinder stains, and axis-cylinder and myelin stains.

B. *Nerve-cells.*

(*c*) Axis-cylinder and protoplasm *stains*, comprising the methylen-blue method and some rather old-fashioned general stains.

(*d*) Axis-cylinder and protoplasm *impregnations*, consisting chiefly of the methods of Golgi (the sublimate method and the three bichromate of silver methods), and the gold methods that have been given in previous chapters.

Nature acknowledges no absolute distinction between a central and a peripheral nervous system; and as, moreover, the chief neurological methods are applicable both to the study of central and of peripheral nerve-tissue, it does not seem advisable to make a formal distinction of that sort here. The three following chapters wear the aspect of being devoted chiefly to the "Central Nervous System" simply because a large proportion of the methods used in the study of nerve-tissue in peripheral organs have already been extensively treated of in the chapters on "Methylen Blue," on "Impregnation Methods," on "Tegumentary Organs," and on "Muscle and Tendon." The reader will kindly bear in mind that a considerable part of the subject properly comprehended under the term "Neurological Methods" is contained in those chapters, which should be referred to in order to complete the account given in the following pages.

The remainder of this chapter will be devoted to the special section methods employed for the central nervous system, a knowledge of which is a necessary preliminary to further study, and to the Cytological Methods of Neurology. Group A of the Anatomical Methods will be given in Chap. XXXII, and Group B in Chap. XXXIII.

For more minute details concerning the dissection and hardening of the voluminous encephala of Man and the larger Vertebrates than can be given here see Mercier, *Les Coupes du Système Nerveux Central* (1894, Paris, Rueff); Dejerine, *Anatomie des Centres Nerveux*, 1895; Bevan Lewis, *The Human Brain; Histological and Coarse Methods of Research*, London, Churchill; and Obersteiner, *Anleitung beim Studium des Baues d. nervösen Centralorgane im gesunden u. kranken Zustande*, Leipzig, Toeplitz. These very welcome additions to the literature of the subject relieve me from the obligation of treating the matter with all the minuteness that might be desired by specialists; the more so as they show that so to treat it would require a volume, not a chapter.

SECTION METHODS.

700. Fixation by Injection.—Fixation, in the proper sense of the word, is of course out of the question in the case of the human subject. But in the case of the lower animals it is possible to introduce fixing liquids into the living nerve-centres by means of injection, thus ensuring a much more rapid penetration of the reagents than can be obtained by simple immersion. This method was, I believe, first suggested by GOLGI (*Arch. Ital. de Biologie,* t. vii, p. 30). He injected 2·5 per cent. solution of bichromate of potash, through the carotid if he wished to limit the hardening to the encephalon, or through the aorta if he desired to fix the spinal cord.

DE QUERVAIN (*Virchow's Archiv,* cxxxiii, 1893, p. 489; *Zeit. f. wiss. Mik.,* x, 4, 1893, p. 507) proceeds as follows with dogs or cats. The subject, lightly chloroformed, is fixed securely on the operating table. A carotid is laid bare and cut through, and a canula fitted with a stopcock is tied both into the central and the distal portion. The cock of the central portion is opened first, and blood is allowed to flow out till no more comes away. Then liquid of Müller, at the temperature of the body, is injected under a moderate pressure through the peripheral canula (therefore towards the brain). This kills the subject at once and begins the fixation. Then more liquid of Müller is injected alternately through the central canula and the peripheral one, until a quantity has been injected equal to the quantity of blood that has been abstracted. For dogs 300 c.c. to 600 c.c. will be required, for cats from one third to one half of that quantity. As soon as the injection has been made, ligatures are placed on the cut ends of the vessel; the encephalon is removed, and is put for some weeks into liquid of Müller kept at 37° C.

MANN (*Zeit. f. wiss. Mik.,* xi, 4, 1894, p. 482) proceeds in a similar manner. He injects through the aorta. Before throwing in the fixing liquid, he injects for about twenty seconds physiological salt solution warmed to 39° C. This washes out the capillaries, and prevents the blood from coagulating there. The fixing solution employed by him consists of saturated solution of corrosive sublimate, warmed to 39° C. After five minutes of injection the brain ought to be fixed throughout. It is removed and put for twelve hours into the same sublimate solution, after which it is either put for permanent preservation into 0·1 per cent. solution of sublimate, or is at once passed through alcohol for imbedding in paraffin.

STRONG (*New York Acad. of Sci.*, January 13th, 1896; *Anat. Anz.*, xi, 21, 1896, p. 655) advises injecting formalin diluted with an equal volume of water into the cephalic vessels until it runs from the cut jugulars. After a few minutes the same quantity is again injected, and once or twice again after a lapse of fifteen to twenty minutes. When only the Golgi method is to be used, an equal volume of 10 per cent. solution of potassium bichromate is to be taken instead of the water. I must say this treatment seems to me very heroic (see § 86).

Hardening.

701. Hardening by the Freezing Method.—This is in many cases a very good method, and in particular may be of service for the histological study of the cortex.

If it be desired to freeze an organ that has been already hardened by reagents, the freezing may be done by means of a freezing mixture of ice and salt; but in this case the preparation should first be penetrated by a mucilaginous or gelatinous freezing mass (§ 177, *et seq.*), in order to avoid the formation of ice crystals in the tissues. But in the case of fresh tissue the ether freezing method is to be preferred. This method allows of rapidly producing any desired degrees of hardness, and maintaining them or allowing them to diminish as occasion may require, and is perhaps the only method by which satisfactory sections of unhardened nerve-tissue can be obtained.

The sections should be floated on to water, treated for a minute on the slide with 0·25 per cent. osmic acid solution, and stained or otherwise treated as desired.

For a detailed description of these manipulations see BEVAN LEWIS's *The Human Brain.*

702. Generalities on Hardening by Reagents.—If large pieces of nerve-tissue are to be hardened, it is necessary to take special precautions in order to prevent them from becoming deformed by their own weight during the process. Spinal cord or small specimens of any region of the encephalon may be cut into slices of a few millimetres thickness, laid out on cotton wool, and brought on the wool into a vessel in which they may have the hardening liquid poured over them. The wool performs two functions: it forms an elastic cushion on which the preparations may lie without being distorted by their own weight; and it allows the reagent to penetrate by the lower surfaces of the preparations as well as by their exposed surfaces. A further precaution, which is useful, is to hang up the preparations, lying on or in the cotton wool, in a glass cylinder or other tall vessel; by hanging them near the top of the liquid the processes of diffusion and the penetration of the reagent are greatly facilitated.

If the preparations are placed on the bottom of the vessel, they should never be placed one on another.

If it be desired to harden voluminous organs without dividing them into portions, they should at least be incised as deeply as possible in the less important regions. It is perhaps better in general not to remove the membranes at first (except the dura mater), as they serve to give support to the tissues. The pia mater and arachnoid may be removed partially or entirely later on, when the hardening has already made some progress.

The *spinal cord*, the *medulla oblongata*, and the *pons Varolii* may be hardened *in toto*. The dura mater should be removed at once, and the preparation hung up in a cylinder-glass, with a weight attached to its lower end. The weight has the double function of preventing any part of the preparation from floating above the level of the hardening liquid (a thing that easily happens where somewhat dense liquids, such as Müller's solution, are used), and of preventing the torsions of the tissues that may otherwise be brought about by the elastic fibres of the pia mater and arachnoid.

The *cerebrum* should be very delicately laid out on a layer of cotton wool, or, if possible, hung up in it. Plugs of the wool should be put into the fissure of Sylvius, and as far as possible between the convolutions. Unless there are special reasons to the contrary, the brain should be divided into two symmetrical halves by a sagittal cut passing through the median plane of the corpus callosum. Betz recommends that after a few hours in the hardening liquid the pia mater should be removed wherever it is accessible, and the choroid plexuses also. I have found this by no means easy, and think it is an operation that can only be recommended for experienced hands.

The cerebellum should be treated after the same manner.

The temperature at which the preparations are kept in the hardening solution is an important point. The hardening action of most solutions is greatly enhanced by heat. Thus Weigert (*Centralb. f. d. med. Wiss.*, 1882, p. 819; *Zeit. f. wiss. Mik.*, 1884, p. 388) finds that at a temperature of from 30° to 40° C. preparatious may be sufficiently hardened in solution of Müller in eight or ten days, and in solution of Erlicki in four days; whilst at the normal temperature two or three times as long would be required.

But it is not certain that this rapid hardening always gives the best definite results. Sahli, who has made a detailed study of the hardening action of chrome salts, is of opinion that it does not, and thinks it ought for this reason to be abandoned (see *Zeit. f. wiss. Mik.*, 1885, p. 3).

On the other hand, the slowness of the action of chromic salts at the normal temperature is such that decomposition may easily be set up in the tissues before the hardening and preserving fluid has had time to do its work. For this reason voluminous preparations that are to be hardened in the slow way should be put away in a very cool place—best of all in an ice-safe.

703. The Reagents to be employed.—The hardening agents most used are the *chromic salts.* Chromic acid was much used at one time, but most workers now agree that its action, though much more rapid than that of the salts, is much more uneven, and frequently causes a disastrous friability of the tissues. Osmic acid is excellent—according to Bevan Lewis it is the best of hardening agents; but its employment is unfortunately very restricted, as it can hardly be used for objects of more than a cubic centimetre in size.

It has already been noted that the liquid of Erlicki has a more rapid action than the other solutions of chromic salts; for this reason it is one of the most commonly employed solutions. Sahli, however (l. c.), after having studied the action of the usual solutions, concludes that the best hardening agent for fresh tissues is *pure bichromate of potash,* in 3 or 4 per cent. solution, the hardening being done in a cold place. And he does not approve of the addition of sodium sulphate (Müller), and rejects the liquid of Erlicki on account of the precipitates it so frequently gives rise to (see § 96).

Obersteiner is of the same opinion, recommending pure bichromate for general hardening purposes; whilst for the study of the most delicate structural relations he recommends fixing in Fol's modification of Flemming's liquid (p. 32) for twenty-four hours, followed by washing with water and hardening in 80 per cent. alcohol.

In view of the slowness of penetration of chromic salts it is often advisable to treat preparations for twenty-four hours or more with alcohol of 80 to 90 per cent. before putting them into the hardening liquid, in or der to avoid maceration of the deeper layers of tissue.

Two recent observers, Fish (*The Wilder Quarter-Century Book*, 1893, p. 335) and Donaldson (*Journ. of Morphol.*, ix, 1894, p. 123; *Journ. Roy. Mic. Soc.*, 1894, p. 642), have made numerous determinations of weight and volume with the object of ascertaining what changes are produced by reagents in encephala of sheep. They have found that bichromate of potash produces a slight increase both of weight and volume, whereas all the other reagents tried produce a diminution of both these factors.

Formaldehyde is a reagent that has been too recently introduced for it to be desirable to enunciate a definitive opinion concerning its fixative and hardening properties. I have quoted in §§ 86 and 109 some authors who have found it advantageous, especially in the form of mixtures with bichromate or other reagents, in the hardening of nervous tissue, and some other formulæ of authors will be given further on. I have already stated (§ 86) that I find that used pure it does not faithfully preserve the minute structure of cells. There is no doubt that it is excellent for the preservation of specimens intended only for macroscopical study, but that is another thing. See further §§ 711 and 765 below.

For the question as to how far certain so-called pathological alterations of ganglion-cells should be attributed to putrefractive changes or to the influence of reagents see *Neurologisches Centralb.* for the years 1884 and 1885.

As to the so-called "pigment spots" produced by the liquid of Erlicki see *supra*, § 96.

704. Strengths of the Reagents.—All hardening reagents (except osmic acid) should at first be taken as weak as is consistent with the preservation of the tissue, and be changed by degrees for stronger.

Osmic acid may be taken of 1 per cent. strength, and will harden small pieces of tissue sufficiently in five to ten days (Exner).

Bichromate of potash should be taken at first of not more than 2 per cent. strength; this is then gradually raised to 3 or 4 per cent. for the cord and cerebrum, and as much as 5 per cent. for the cerebellum. Obersteiner begins with 1 per cent., and proceeds gradually during six to eight weeks to 2 or 3 per cent. (This is at the normal temperature ; at a temperature of 35° to 45° C. the hardening can be got through in one or two weeks.)

Bichromate of ammonia should be taken of half the strength recommended for bichromate of potash, or even weaker at first ; it may be raised to as much as 5 per cent. for cerebellum towards the end of the hardening.

Chromic acid is not much used alone (see § 88). It forms part of some of the mixtures mentioned below. A very little chromic acid (say one to two drops of 1 per cent. solution for each ounce) added to bichromate solution will do no harm, and will quicken the hardening.

Nitric acid has been and still is employed in strengths of 10 to 12 per cent., and gives particularly tough preparations.

Neutral acetate of lead in 10 per cent. solution affords an excellent preservation of ganglion-cells, according to ANNA KOTLAREWSKI (see *Zeit. f. wiss. Mik.*, iv, 3, 1887, p. 387).

TRZEBINSKI (*Virchow's Arch.*, 1887, p. 1 ; *Zeit. f. wiss. Mik.*, iv, 4, 1887, p. 497) finds that, as regards the faithful preservation of ganglion-cells (of the spinal cord of the rabbit and dog), the best results are obtained by hardening for eight days in 7 per cent. solution of *corrosive sublimate*, followed by hardening in alcohol containing 0·5 per cent. of iodine.

DIOMIDOFF (*ibid.*, p. 499) also obtained very excellent results by hardening small pieces of brain (as suggested by GAULE, OGATA, and BECHTEREFF) for from five to nine days (not more in any case) in 7 per cent. sublimate solution, and then putting the tissues for twenty-four hours into 50 per cent. alcohol, and for the same time into 70 per cent. and 96 per cent. alcohol successively. (This process produces artificial "pigment spots," similar to those produced by solution of Erlicki ; they may be dissolved out by prolonged treatment with warm water, or in five minutes by strong solution of LUGOL.) The tissues are of a good consistence for cutting. Chloride of zinc has been recommended for some purposes (see below, §§ 710, 711).

The next following paragraphs give in detail some methods of hardening recommended by some of the most competent workers.

705. BETZ'S **Methods** (*Arch. f. mik. Anat.*, 1873, p. 101).—The *spinal cord, medulla oblongata,* and *pons Varolii* are treated as follows :—The dura mater is removed, and they are hung up in a cylinder containing 75 to 80 per cent. alcohol, to which is added enough iodine to produce a light brown coloration. After from one to three days the preparation will be found to be somewhat surface-hardened ; it is taken down, and the pia mater and arachnoid are removed. If the pia mater does not come away completely enough the preparation is put back for some days into the alcoholic iodine. The membranes having been removed, the preparation is put back into the original fluid, which is found to have become colourless owing to absorption of the iodine by the tissues. Fresh quantities of a strong solution of iodine in alcohol are from time to time added to the liquid in order to keep it at its original strength of iodine (as shown by the colour). If the membranes have been carefully removed, it will be found that after about six days the preparation ceases to take up further quantities of iodine. The preliminary hardening may now be considered complete.

The preparation is now brought into a 3 per cent. solution of bichromate of potash. (A small weight is attached to it to prevent any portion of it from floating above the surface of the liquid. After a day or two it will have lost much of its alcohol, and will sink to the bottom of the vessel, which is equally undesirable; this must be watched for, and the preparation hung up or otherwise supported.) The vessel is put away *in a cool place.* As soon as a brown turbidity is seen in the liquid, together with a brown deposit on the preparation, the hardening may be considered to be complete. The preparation must be at once washed with water, and put away until wanted in a ½ to 1 per cent. solution of bichromate.

Cerebellum.—Must be quite fresh, and before placing in the iodine the membranes and vessels must as far as possible be very carefully removed. (If the pia mater does not come away freely the organ must be macerated for a few hours in iodine solution in which other preparations have been kept, and which is diluted before using for this purpose.) The membranes having been removed, the cerebellum is placed (supported on cotton wool, with which the different organs are so propped up as to preserve their natural position) in solution of iodine for two or three days, and fresh iodine solution frequently added.

The pia mater is now removed from the rest of the preparation, which is put back for seven to fourteen days into the iodine solution. If at the expiration of this time it be found that the cerebellum can be supported on the finger by the vermiculus alone without bending, the preliminary hardening is complete, and it is brought into a 5 per cent. solution of bichromate, where it remains until fit for cutting.

Cerebrum.—The cerebrum is divided into two halves along the median line of the corpus callosum, and put into the iodine solution. After a few hours the pia mater is removed from the fissure of Sylvius and from the corpus callosum, and if possible the choroid plexus is removed likewise.

The preparation is now put away in the iodine solution in a cool place (in summer in a cool cellar), and fresh iodine added as soon as the liquid is seen to lose colour (which must be watched for). After twenty-four to forty-eight hours the remaining pia mater is carefully removed by means of scissors and forceps from the fissures and convolutions, and one half-volume of fresh iodine solution is added to the liquid. (To facilitate the penetration of the liquid, wads of cotton wool are stuffed into the fissure of Sylvius and between the "operculum" and the central lobe [" *Centrallappen* "], in the direction of the descending cornu, and between the convolutions.) After twenty-four to seventy-two hours the brain is brought into fresh solution of iodine in 70 per cent. alcohol, where it remains until the hemispheres are hard enough to be supported on two fingers without bending. (This will not be before ten to fourteen days.) It is then put into 4 per cent. solution of bichromate and left to acquire its definitive hardness. If an excessive brown deposit make its appearance, and the brain be found notwithstanding to be not hard enough for cutting, it must be rinsed with water and the bichromate solution changed. When ripe for cutting the brain ought to show an almost equal intensity of yellow-brown stain over the whole surface of a cut made through the total thickness of a hemisphere.

Brains that are not fresh require for hardening longer time and stronger alcohol.

The methods of Betz are particularly adapted to the hardening of voluminous specimens, and of tissues that are in a state of post-mortem softening.

706. Cerebrum (BEVAN LEWIS, *The Human Brain*, p. 102).—Methylated spirit, twenty-four hours in a cool place. Müller's solution, three days in a cool place. Then change the liquid;

and after three days more change it again, or, preferably, substitute a 2 per cent. solution of potassium bichromate. At the end of the second week a solution of double the strength may be added; and if at the termination of the third week the mass is still pliable, and of the consistence of ordinary rubber, it as yet unfit for section cutting, and the reagent should be replaced by a solution of chromic acid.

707. Brain (HAMILTON, *Journ. of Anat. and Physiol.*, 1878, p. 254).—Take a fresh brain and make a series of incisions into different parts, still keeping everything *in situ;* or slice it into any number of segments about one inch thick, but of the whole length or breadth of the organ, as may be desired. Do *not* remove the membranes; they form a protection for the superficial layers, and do not interfere with the hardening process. The large segments are placed flat in a large vessel padded with cotton; do not put them one above the other. Cover them with the following fluid:

Müller's fluid . . .	3 parts.
Methylated spirit . .	1 part.

(Heat is evolved on mixing these liquids, and the mixture must be allowed to cool before pouring it over the brain tissue.) Put the preparations away in an ice-safe. Turn the segments over next day. Change the solution in a fortnight or three weeks; or if on examining a section of one of the pieces it is found that the hardening reagent has penetrated to the interior, they may be at once removed to the following mixture:

Bichromate of ammonia . .	1 grm.,
Water	400 c.c.,

in which they remain for one week. Then change the solution to one of 1 per cent. for one week; and let this be followed by a solution of 2 per cent. for another week, or longer if required. The pieces will now be sufficiently hard for cutting; they may be kept permanently in solution of chloral hydrate, twelve grains to the ounce.

Probably the chloral hydrate serves to attenuate the yellow coloration produced by the chromic liquids.

This is a process particularly adapted to the preparation of *large* segments of brain. The consistence is very tough and firm.

708. Entire Encephalon (Deecke, *Journ. Roy. Mic. Soc.*,1883, p. 449).—To harden the entire brain so that the inside and the outside shall be hardened equally and properly, Dr. Deecke finally adopted bichromate of ammonia in $\frac{1}{2}$ to 1 per cent. solution, according to the consistence of the brain. If it happens to be soft he adds say $\frac{1}{6}$ to $\frac{1}{10}$ per cent. of chromic acid to the solution, and always $\frac{1}{6}$ to $\frac{1}{4}$ of the whole volume of alcohol. It is then placed in a refrigerator and the fluid changed frequently. After a month add a little more alcohol from week to week until the alcohol is 90 per cent. This is changed as often as it is discoloured. The treatment requires from twelve to eighteen months.

709. Encephalon (M. Duval, Robin's *Journal de l'Anatomie*, 1876, p. 497).—*First Method.*—Place the fresh tissues in solution of bichromate of potash 25, water 1000; change the liquid after the first twenty-four hours, and again after three or four days. After two or three weeks place the preparations in chromic acid of 3 per 1000, change the liquid every day for the first week, and after that every eight days until the middle of the second month, after which time it is no longer needful to change the liquid. The preparations must remain at least two months in the chromic acid; the longer they remain in it the better. A few fragments of camphor should be added to the liquid in order to prevent the growth of mould.

710. Encephalon (Fish, from an interesting paper on "Brain Preservation" in *The Wilder Quarter-Century Book*, 1893, p. 393). The following is said to be not ideal in its effects, but to answer the requirements of economy, fixation of the structural elements, differentiation of tissue, firmness of texture, and rapidity of action:

Water	400 c.c.
95 per cent. alcohol . .	400 ,,
Glycerin	250 ,,
Zinc chloride	20 grms.
Sodium chloride . . .	20 ,,

Immerse in this, filling the cavities of the brain with it, and if practicable also injecting the blood-vessels with it, for about three days, then transfer for a week or more to a mixture of equal parts of the fluid and 70 per cent. alcohol, and finally store in 90 per cent. alcohol.

711. Formaldehyde.—The liquids employed by Strong and Dorig are given below in § 765. See also § 109.

Weigert (*Beitr. zur Kenntn. d. normalen menschlichen Neuroglia*, 1895, quoted from *Neurol. Centralb.*, 1895, p. 1146) puts portions of material of not more than half a centimetre in thickness for four days into a 4 per cent. solution of formol (by which is presumably meant commercial formol diluted with 9 volumes of water).

Marcus (quoted from Fish, see below) recommends hardening the spinal cord for two or four weeks in a ½ per cent. solution of formalin, then small pieces one half-centimetre thick are cut out and placed in Müller's fluid for a week in an oven at 37° C.

Van Gieson (*Anat. Anz.*, x, 1895, p. 494) states that he obtained good results by using "solutions of formalin of 4, 6, and 10 per cent.," followed by 95 per cent. alcohol. Myelin was found to be well preserved and to give the characteristic blue reaction with Weigert's hæmatoxylin (the 1885 method), just as if a chrome salt were present.

Lachi (cf. *Zeit. f. wiss. Mik.*, xii, 1895, p. 32) states that he has had good results with "20 per cent. solutions of formol."

Fish (*Proc. Amer. Mic. Soc.*, xvii, 1895, p. 319) recommends the following formula:

Water	2000 c.c.
Commercial formalin . .	50 ,,
Sodium chloride . . .	100 grms.
Zinc chloride . . .	15 ,,

Brains should be left in this mixture for a week or ten days or more, then transferred to a 2·5 per cent. solution of formalin (water 2000 c.c., formalin 50 c.c.), in which they may remain indefinitely if the jar be kept tightly covered. After an immersion of two weeks in these solutions a human brain lost only 6·8 per cent. of its weight; but after an immersion for a similar period of eight days in 50 per cent. alcohol and eight days in 70 per cent. alcohol it was found to have lost 22 per cent. of its first weight.

Parker and Floyd (*Anat. Anzeiger*, Bd. xi, 1895, No. 5,

p. 156) find that a "2 per cent. solution of formol," by which is meant a mixture of two volumes of formol with 98 of water, will harden a sheep's brain in a week or ten days in a satisfactory manner as regards consistency, but with a marked increase of volume, which may amount to as much as 40 per cent. To obviate this they advise a mixture of—

Alcohol 95 per cent. . . .	6 volumes,
Formol 2 per cent. (the above mixture)	4 ,,

which has the same excellent and rapid hardening qualities and gives only a hardly perceptible increase of volume. Brains may be kept for months in the mixture (*ibid.*, 1896, p. 568).

712. Berkley, cortex of cerebellum of the dog (cf. *Zeit. f. wiss. Mik.*, x, 3, 1893, p. 388). Liquid of Flemming, twenty-four to thirty-six hours; absolute alcohol; celloidin.

713. Nervous Centres of Reptiles and Amphibia (Mason, *Central Nervous System of Certain Reptiles*, &c.; Whitman's *Methods*, p. 196).—Iodised alcohol, six to twelve hours; 3 per cent. bichromate, with a piece of camphor in the bottle, and to be changed once a fortnight until the hardening is sufficient (six to ten weeks).

Burckhardt (*Das Centralnervensystem von Protopterus*, Berlin, 1892; *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 347) recommends a liquid composed of 300 parts of 1 per cent. chromic acid, 10 parts of 2 per cent. osmic acid, and 10 parts of concentrated nitric acid, in which brains of Protopterus are hardened in twenty-four to forty-eight hours.

Fish (*Journ. of Morphol.*, x, 1, 1895, p. 234) employed for the encephalon of *Desmognathus fusca* a mixture of 100 c.c. of 50 per cent. alcohol, 5 c.c. of glacial acetic acid, 5 grms. of corrosive sublimate, and 1 grm. of picric acid, fixing for twelve to twenty-four hours, and passing through the usual alcohols.

Imbedding and Cutting.

714. The Methods of Imbedding.—The paraffin infiltration method can only be used for the smaller objects of this class. Human spinal cord (which is quite at the upper limit as regards size) can be properly penetrated with paraffin by taking the precaution of first cutting it up into slices of not more than a few millimetres—preferably not more than one—in thickness. The largest objects of this class, such as entire hemispheres of man, cannot be properly penetrated by any known imbedding mass; and the anatomist must be content with simple superficial imbedding—a proceeding which is

UNIVERSITY

here of the greatest service. For intermediate objects—those whose size varies between that of a small nut and a walnut—it appears to me that no hesitation as to the proper course is possible; such objects should be treated by the collodion method, which is at once the safest, the most convenient, and the most advantageous as regards the ulterior treatment of sections.

Imbedding is not a necessary process. Sections can be obtained from any part of the central nervous system without imbedding. The material should be very well hardened, and a suitable piece should be glued on to a piece of wood or cork by means of a rather thick solution of gum arabic. As soon as it begins to stick to the support the whole is thrown into 80 per cent. alcohol to harden the joint, after which it may be fixed in the object-holder of the microtome and cut.

If the collodion method has been taken a difficulty may arise. It may be found that, notwithstanding every precaution, the collodion has not thoroughly penetrated the tissues. Good sections may, however, still be obtained by Duval's method of collodionising the sections. The cut surface of the tissue is dried by blowing on it, and is covered with a thin layer of collodion laid on it with a brush. As soon as this layer has somewhat dried, which happens very rapidly, a section is cut, and the cut surface collodionised as before, and so on for each section. This process gives very good results, and may be advantageously employed even with material that has been successfully imbedded, as it gives a better consistency to the tissue, and enables thinner sections to be obtained (VAN GEHUCHTEN, *in litt.*).

BEVAN LEWIS recommends the ether freezing method for fresh brain. For hardened brain he recommends some of the old-fashioned wax-and-oil and other fatty mixtures, a doctrine at which I am surprised.

HAMILTON recommends freezing in the gum and syrup mass given above, § 178. He also recommends a method of penetrating with collodion, which is hardened in the usual way, the hardened mass being cut with an ice-and-salt freezing microtome (see *Journ. of Anat. and Physiol.*, 1887, p. 444; or *Journ. Roy. Mic. Soc.*, 1888, p. 1051).

GOODALL's Rapid Method for preparing Spinal Cord (*Brit. Med. Journ.*, May, 1893, p. 947; *Journ. Roy. Mic. Soc.*, 1893, p. 405).—Cut sections of fresh tissue with a freezing microtome; float them on to water, and as soon as possible drain them and float them on to pyridin. After a quarter of an hour wash in water; stain with 0·25 per cent. aqueous solution of anilin blue-black, followed by picro-carmine; dehydrate and clear in pyridin; mount in balsam thinned with pyridin. See also *ante*, § 107.

BENDA (*Centralb. f. Allgem. Path. u. path. Anat.*, vi, 1895, p. 803) finds there is great advantage in using formaldehyde for removing the alcohol from hardened tissues before freezing. Small pieces of material should be left for from a quarter of an hour to several hours in 1 per cent. formaldehyde solution (2½ per cent. of commercial formalin), and washed in distilled water and frozen therein. The tissues after thawing are not found to be brittle, as is the case with those that have not been treated with formaldehyde, but have a peculiar tough soapy consistency.

For sections of entire human brain, DEECKE (l. c., § 708) proceeds as follows:—The brain to be cut is placed upon the piston of the microtome (a Ranvier model) and held *in situ* by several pieces of soft cork. It is then imbedded in a cast of paraffin, olive oil, and tallow, which, after it has become hard, is held in position by a number of small curved rods attached to, and projecting upwards from, the piston to the height of about an inch. Before cutting, and as it proceeds, the cast is carefully removed from around the specimen to the depth of about half an inch (which is easily done by the use of a good-sized carpenter's chisel), so that the knife never comes in contact with the cast.

Cutting is done under alcohol, the entire microtome being immersed in a copper basin. The sections are floated, with the aid of a fine camel-hair brush, on to sheets of glazed writing-paper. They are removed thereon successively into staining, washing, and clearing fluids. After clearing, they are brought on the paper on to a slide, and the paper is gently pulled away from them; they are then mounted in chloroform- or benzol-balsam.

It should be noted that the membranes should *not* be removed from the brain; they present no obstacle to cutting if this is done with a slight sawing movement, or with a series of short cuts, instead of one sweep of the knife. By this plan the sections are much more perfect and uniform in thickness, and the loss in a series of from four to five hundred to the inch through the entire cerebrum of man may not amount to more than 2 or 3 per cent.

OSBORN (*Proc. Acad. Nat. Sci. Philadelphia*, 1883, p. 178, and 1884, p. 262: WHITMAN'S *Methods*, p. 195) found advantage in employing Ruge's egg-mass (for the brain of Urodela). He recommends that the mass be injected into the ventricles.

The DEJERINES (*Anat. des Centres Nerveux*, t. i, 1895, p. 29) have found advantage in the employment of both collodion and paraffin at the same time, for cutting entire hemispheres. The preparation is thoroughly impregnated with the collodion, which after hardening should form a wall of a couple of centimetres in thickness all around it. On the lower surface of this wall of collodion, incisions of about one centimetre in depth are made, running in various directions. The whole is then plunged into the cylinder of a Gudden microtome containing melted paraffin, and oriented according to the direction in which it is desired to make the sections. It is not necessary that the preparation should be entirely covered by the paraffin; it is sufficient that it should plunge therein for about one third of its depth.

The paraffin penetrates into the cuts that have been made in the collodion, and after it has solidified is found to hold the preparation firmly. A very considerable quantity of paraffin is required for this operation, and as in drying it undergoes great shrinkage, it is well to wait for twenty-four hours before making the sections.

Feist (*Zeit. f. wiss. Mik.*, viii, 4, 1892, p. 492) gives a useful hint for marking the right and left sides of spinal cord. He imbeds with each segment of the cord a small cylinder (of about 1 square millimetre in section) of hardened liver, stuck vertically in the imbedding mass (either celloidin or paraffin) against the side of the cord that it is desired to mark.

Cytological Methods.

715. General Cytological Methods.—For the general principles of cytological research as applicable to nervous elements the reader is referred to the chapter on "Cytological Methods." The following paragraphs are of a more special nature.

(*a*) *Nerve-cells.*

716. Nissl's **Fuchsin Method** (*Neurol. Centralb.*, quoted from Mercier's *Coupes du Système Nerveux Central*, 1894, p. 183).—Perfectly fresh material is to be cut into pieces of not more than one cubic centimetre in size. These are put for two days into a "chromic solution in 70 per cent. alcohol" (strength not stated). They are then transferred for five days to absolute alcohol, and may then be sectioned.

The sections are brought into a saturated solution of fuchsin contained in a deep watch-glass, only one section being treated at a time. The watch-glass is then warmed over a flame, care being taken not to overheat the preparation, until vapours begin to be given off from the liquid. Then the section is removed and plunged for one to two minutes into absolute alcohol. It is then got on to a slide and covered abundantly with clove oil, in which it remains until no more colour is given off. It is then drained, washed once with fresh clove oil, drained and mopped up, and mounted in balsam.

Nuclei and the chief protoplasmic processes are demonstrated, as also neuroglia cells, but not myelin.

717. NISSL's **Methylen-blue Method** (*Neurol. Centralb.*, 1894, p. 508).—Fresh material is hardened in 96 per cent. alcohol, and sectioned. The sections are brought into a watch-glass with the following stain:

Methylen blue (Methylenblau pat.)	3·75	parts.
Venice soap	1·75	,,
Distilled water	1000·0	,,

The watch-glass is warmed over a flame to about 65° to 70° C., till bubbles are given off which burst at the surface of the liquid. The sections are then brought into a mixture of ten parts of anilin oil with 90 parts of 96 per cent. alcohol, and are differentiated therein until colour is no longer given off from them. They are got on to a slide, dried with filter-paper, cleared with oil of cajeput, dried again with filter-paper, treated with a few drops of benzin, and mounted in benzin-colophonium.

Professor VAN GEHUCHTEN, to whom I am indebted for this process, obtains the same results in a more commodious manner. Sections are cut by the paraffin method, and fixed on a slide by the distilled-water method (see § 186). The paraffin is removed by means of xylol, the slide is passed rapidly first through absolute alcohol, then through 80 per cent. alcohol, and is put into a dish with Nissl's staining mixture. The whole is put for five or six hours into a stove kept at about 35° to 40° C. Differentiation is performed as in Nissl's process, and the preparation is mounted in xylol-damar. In this way a considerable series of sections can be treated at one time.

718. ROSIN's **Method** (*Neurol. Centralbl.*, xii, 1893, p. 1; *Zeit. f. wiss. Mik.*, xii, 1, 1895, p. 77) consists in staining with the Ehrlich-Biondi mixture.

719. The methods of REHM (*Münchener med. Wochenschr.*, 1892, No. 13; *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 390) are as follows:—Sections are stained for a few minutes in concentrated aqueous solution of Congo, washed in alcohol, and treated for ten minutes, until they become blue, with alcohol acidulated with hydrochloric or nitric acid, then cleared with origanum oil and mounted. A sharp axis-cylinder stain with considerable richness of other detail.

But REHM prefers the following simple process:—Sections of alcohol-hardened material are placed for one to two days in an aqueous solution of hæmatoxylin of 0·5 per cent. strength, then washed out in aqueous solution of carbonate of lithia (strength not given) until no more colour comes away from them, dehydrated and mounted. Axis-cylinders, cells, and processes, grey-black. The sections may be after-stained for a few minutes with 0·1 per cent. aqueous solution of Bismarck brown.

(*b*) *Nerve Fibres.*

720. Structure of Medullated Nerve.—In order to demonstrate the axis-cylinder and the sheath of Schwann, the myelin may be removed. This may be done by boiling in caustic soda, and then neutralising; by boiling in a mixture of absolute alcohol and ether, and adding caustic soda; by boiling in glacial acetic acid; by boiling in fuming nitric acid, and adding caustic potash; or by treating with eau de Javelle; or (VAN GEHUCHTEN, *in litt.*) the myelin may be extracted in the cold by leaving the nerves for some time in a mixture of alcohol and ether.

721. KUPFFER'S **Method** (*Sitzb. math. phys. Kl. k. Bayr. Akad. Wiss.*, 1884, p. 446; *Zeit. f. wiss. Mik.*, 1885, p. 106).—A nerve is stretched on a cork and treated for twenty-four hours with 0·5 per cent. osmic acid. It is then washed in water for two hours and stained for twenty-four to twenty-eight hours in saturated aqueous solution of Säurefuchsin; after which it is washed out for from six to twelve hours (not more in any case) in absolute alcohol, cleared in clove oil, imbedded in paraffin, and cut. Sections are said to show the axis-cylinder as a bundle of fibrils (stained red) floating in an albuminous liquid.

722. Neuroceratin Structures (GALLI, *Zeit. f. wiss. Mik.*, iii, 1, 1886, p. 467).—An ischiatic nerve is excised, and fixed and hardened for eighteen to twenty minutes in solution of Müller. Small portions of the nerve are then further treated for one or two days with solution of Müller diluted with 2 parts of water, then for a quarter of an hour with glycerin containing 1 or 2 drops of glacial acetic acid for each cubic centimetre, and finally (without previous washing with water) are stained for fifteen to twenty minutes in aqueous solution of China blue (the best China blue for this purpose is that supplied by the Badische Anilin- und Soda-Fabrik, at Stuttgart). They are washed out in alcohol, cleared in essence of turpentine, and mounted in dammar.

For the results see the paper and the plate, l. c.

PLATNER (*Zeit. f. wiss. Mik.*, vi, 2, 1889, p. 186) obtains a specific stain of the neuroceratin framework of medullated nerve in the following way:—Small nerves are fixed and hardened for several days in a mixture of 1 part of Liq. Ferri Perchlor. (Ph. G., ed. 2) and 3 to 4 parts of water or alcohol. They are then to be washed out in water or alcohol till no traces of iron remain in them (the reaction of the washings with rhodanide of potassium being a good test of this), and are stained for several days or weeks in a concentrated solution of "Echtgrün" (dinitrosoresorcin, not "dinitroresorcin," as erroneously in Platner's paper) in 75 per cent. alcohol; after which they are dehydrated, imbedded, and sectioned.

See also the papers of Gedoelst in *La Cellule,* iii, 1887, p. 117, and v, 1889, p. 126 (good details of digestion methods); also the report in *Zeit. f. wiss. Mik.,* vii, 1, 1890, p. 57.

723. Other Methods.—Ranvier, *Traité,* p. 718, *et. seq.*; Rezzonico, *Arch. per le Sci. Med.,* 1879, p. 237; Tizzoni, *ibid.,* 1878, p. 4 (a process of boiling in chloroform for an hour or two, then staining and mounting in glycerin); Boveri, *Zeit. f. wiss. Mik.,* iv, 1, 1887, p. 91; Jakimovitch, *Journ. de l'Anat.,* xxiii, 1888, p. 142, or *Zeit. f. wiss. Mik.,* v, 4, 1888, p. 526 (instructions for impregnating the axis-cylinder with silver, followed by reduction in formic acid and amyl alcohol); Schiefferdecker, in Behrens, Kossel, u. Schiefferdecker, *Das Mikroskop,* Bd. ii, p. 227; Huber, *Zeit. f. wiss. Mik.,* x, 3, 1893, p. 394 (stains with Benda's safranin and Lichtgrün); Rabl, *ibid.,* xi, 1, 1894, p. 42 (the lines of Frommann are artefacts due to the silver nitrate); Fischel, *ibid.,* p. 48 (similar conclusion); Tirelli, *ibid.,* xi, 3, 1894, p. 391; Segall, *Journ. de l'Anat.,* xxix, 1893, p. 586.

CHAPTER XXXII.

NEUROLOGICAL METHODS—NERVE-FIBRE STAINS (WEIGERT AND OTHERS).

a. Myelin Stains.

724. Introduction.—The most important of the methods for the study of tracts of medullated nerve-fibres are the hæmatoxylin methods of WEIGERT.

There have been in all three methods of WEIGERT; the 1884 method, the 1885 method, and the 1891 method. The ordinary methods of staining with hæmatoxylin depend on the employment of an aluminium lake of hæmatoxylin. Weigert's method depends on the formation of another lake, a chromium or copper lake. In consequence of the formation of these lakes hæmatoxylin acquires the property of staining the myelin of nerves in a quite specific way.

In Weigert's process the formation of these lakes takes place in the tissue itself. The details of the process have been considerably modified, both by other workers and by Weigert himself. The 1884 method (*Fortschr. d. Med.*, 1884, pp. 113, 190; *Zeit. f. wiss. Mik.*, 1884, pp. 290, 564), which depends on the formation of a chrome lake, may be considered to be superseded. Not so the two others, which depend on the formation of a copper lake.

725. WEIGERT'S **1885 Method** (*Fortschr. d. Med.*, 1885, p. 136; *Zeit. f. wiss. Mik.*, 1885, pp. 399, 484).—The tissues are to be hardened in bichromate of potash (the solutions of Müller or Erlicki will do as well, so far as I know). The hardening need only be carried to the point at which the tissues have acquired a *brown*, not a *green* coloration (but green tissues may be used, provided they have once passed through the brown stage). The preparation is then (but this is not necessary) imbedded by infiltration with celloidin, and the celloidin block fastened on cork and hardened in the usual way. The hardened block is put for one or two days into saturated solution of neutral acetate of copper diluted with one volume of water, the whole being kept at the temperature of an incubating stove. By this treatment the tissues become green, and the celloidin bluish green. The mordantage of the tissues is now terminated, and the preparation may be kept, till wanted for sectioning, in 80 per cent. alcohol.

Sections are made with a knife wetted with alcohol, and are brought into a stain composed of—

Hæmatoxylin	0·75 to 1 part.
Alcohol	10 parts.
Water	90 ,,
Saturated solution of lithium carbonate	1 part.

(A trace of any other alkali may be added in the place of lithium carbonate. The object of adding a little of some base is to "ripen" the hæmatoxylin solution.)

The sections remain in the stain for a length of time that varies according to the nature of the tissues:—Spinal cord, two hours; medullary layers of brain, two hours; cortical layers, twenty-four hours.

They are then rinsed with water, and brought into a decolourising solution composed of—

Borax	2·0 parts.
Ferricyanide of potassium	2·5 ,,
Water	200·0 ,,

They remain in the solution until they are decoloured to the right degree—that is, until complete differentiation of the nerves (half an hour to several hours)—and are then rinsed with water, dehydrated with alcohol, and mounted in balsam. They may be previously stained, if desired, with alum-carmine for the demonstration of nuclei.

For very difficult objects, such as pathological nerves, the decolouring solution should be diluted with water, and the immersion in it prolonged. Gelpke (*Zeit. f. wiss. Mik.*, 1885, p. 489) states that for transverse sections of atrophied nerves the solution should be diluted with fifty volumes of water, and the immersion be prolonged to twelve hours at the least; for longitudinal sections it should be diluted with ten volumes of water.

The process is applicable to tissues that have been hardened in alcohol or in any other way, provided that they be put into a solution of a chromic salt until they become brown, before mordanting them in the copper solution.

As above stated, it is not necessary that the mordantage be done in bulk with tissues imbedded in celloidin. Max Flesch (*Zeit. f. wiss. Mik.*, iii, 1, 1886, p. 50) finds that this practice is unfavorable to subsequent staining with other reagents than hæmatoxylin, and prefers (following Lichtheim) to make the sections first, bring them on closet-paper into the mordant, and after mordanting bring them on a spatula into 70 per cent. alcohol, and thence into the stain.

In the process given above, a copper lake is formed in the tissues. In the earlier form of the process the mordantage with the copper salt was omitted, and the stain depended on the formation in the tissues of a chromic lake. The results were not quite so good, and the process may be taken to be superseded by the copper process.

If very many large sections have to be prepared, and if the staining solution be thrown away after using, the process may be found somewhat expensive. The following method for regenerating the staining solution is given by Fanny Berlinerblau (*Zeit. f. wiss. Mik.*, 1886, p. 50):—About 2·5 to 5 per cent. of baryta water is added to the used solution; it is well shaken and allowed to stand for twenty-four hours; carbonic acid (obtained from the action of crude hydrochloric acid on marble) is led through it, it is allowed to stand for twenty-four hours more, and then filtered.

Paneth (*ibid.*, 1887, p. 213) makes the stain with extract of logwood instead of pure hæmatoxylin. One part of commercial extract of logwood is dissolved in 90 parts of water and 10 of alcohol. To the filtered solution is added 8 drops of concentrated solution of lithium carbonate for each 100 c.c. Sections require from eighteen to twenty-four hours in the stain at the normal temperature.

Breglia (*ibid.*, vii, 2, 1890, p. 236; see also *Journ. Roy. Mic. Soc.*, 1890, p. 817) stains with liquid extract of logwood or Pernambuco wood, prepared by extracting 7 to 10 grms. of the wood for five or six days with 100 c.c. of alcohol of 90 to 95 per cent.

The results obtained by Weigert's method are most splendid. The blue-black nerves stand out with admirable boldness on a golden ground. The method is applicable to the study of peripheral nerves as well as to nerve-centres, and is likely to be of great utility in Vertebrate embryology.

Nerve-tissue is not the only tissue stained by the process, which can be usefully applied to lymphatic glands and to skin (see Schiefferdecker, in *Anat. Anz.*, ii, 1887, p. 680).

726. Weigert's **1891 Method** (*Deutsche med. Wochenschr.*, 42, 1891, p. 1184; *Zeit. f. wiss. Mik.*, viii, 3, 1891, p. 392).—The material is to be hardened in bichromate and imbedded in celloidin in the usual way. The hardened blocks of celloidin are brought into a mixture of equal parts of a cold

page 131 for [illegible]

saturated solution of neutral acetate of copper and 10 per cent. aqueous solution of potassio-tartrate of sodium ($C_4H_4O_6KNa + 4H_2O$, salt of Seignette). They are left in the mixture for twenty-four hours in an incubator. (Large specimens [Pons] will require forty-eight hours, the mixture being changed for fresh at the end of twenty-four hours.) They are then brought for twenty-four hours into aqueous solution of neutral acetate of copper, either saturated or diluted with 1 volume of water, being kept as before in the incubator. They are then rinsed with water and brought into 80 per cent. alcohol, in which they may either remain till wanted or be cut after half an hour.

The sections are stained for from four to twenty-four hours at the temperature of the room in a freshly prepared mixture of 9 vols. of (A) a mixture of 7 c.c. of saturated aqueous solution of carbonate of lithium with 93 c.c. of water, and 1 vol. of (B) a solution of 1 grm. of hæmatoxylin in 10 c.c. of alcohol (A and B may be kept in stock, but A must not be too old). The sections must be loose ones, not such as have been seriated in celloidin, and must not be thicker than 0·025 mm. The stain is poured off and the sections are washed in several changes of water poured on to them. They are then treated with 90 per cent. alcohol followed by carbolic-acid-and-xylol mixture (for a short time only), or by a mixture of 2 parts of anilin oil with 1 of xylol, then pure xylol and xylol balsam (not chloroform balsam, which injures the stain).

Medullated fibres dark blue on a light, sometimes rosy ground. If it be wished to have the ground particularly colourless, take instead of the second wash-water a mixture of $\frac{1}{5}$ to $\frac{1}{2}$ volume of common (not glacial) acetic acid with 100 volumes of water. Thick sections or series in celloidin require a special differentiation. They may be differentiated either with the above-mentioned acetic acid mixture, or in the usual borax-ferricyanide mixture diluted with water. In the latter case the ground will be yellow.

If the impregnation with the copper be imperfect (as, for instance, may happen if the treatment with the copper salt be performed at the normal temperature instead of in an incubator) some instructive differentiations of ganglion-cells may be obtained, the processes of the cells of Purkinje in the cerebellum, for instance, being very sharply brought out;

but such preparations have a tendency to after-blackening, which does not happen with those that have been thoroughly impregnated with the copper.

The advantages of the improved method are that differentiation after staining is not necessary; that the annoying precipitates formed on the surface of the preparations by the copper in the old method do not appear; that the divers manipulations are simpler and easier; the preparations are equal in beauty to those of Pal, and can be obtained with greater certainty.

Since the first publication of this method, it has been discovered (WEIGERT, *Ergebnisse der Anat.*, iii, 1894, p. 21) that preparations made as above, without differentiation in the ferricyanide liquid, *do not keep well.* Weigert therefore now advises that they be mordanted as above with salt of Seignette, which has the advantage of preventing the formation of precipitates on the surface of the preparations, but *that they be also differentiated in the ferricyanide,* as in the 1885 method.

Modifications of Weigert's Method.

727. PAL'S **Method** (*Wien. med. Jahrb.*, 1886; *Zeit. f. wiss. Mik.*, iv, 1, 1887, p. 92; *Med. Jahrb.*, 1887, p. 589; *Zeit. f. wiss. Mik.*, 1888, p. 88).—This is a *chrome-lake* process. You proceed at first as in Weigert's process, but *omitting the copper bath,* and you stain as in Weigert's process. After staining in the hæmatoxylin solution the sections are washed in water (if they are not stained of a deep blue a trace of lithium carbonate must be added to the water). They are then brought for twenty to thirty seconds into 0·25 per cent. solution of permanganate of potash, rinsed in water, and brought into a decolouring solution composed of—

Acid. Oxalic. pur. . . .	1·0
Potassium Sulphite* (Kalium Sulfurosum [SO_3K_2]) . .	1·0
Aq. Dest.	200·0

In a few seconds the grey substance of the sections is decolourised, the white matter remaining blue. The sections should now be well washed out, and may be double-stained with Magdala red or eosin, or (better) with picro-carmine or acetic-acid-carmine.

---

* Not "sulphide," as erroneously given in MERCIER'S *Les Coupes du Système Nerveux Central*, p. 190.

For further details as to the somewhat elaborate minutiæ of the process see the papers quoted, or BEHRENS, KOSSEL, and SCHIEFFERDECKER'S *Das Mikroskop*, i, p. 199.

PAL'S process gives more brilliant results than that of Weigert, the ground of the preparations being *totally colourless.* But it has a defect; it is less certain, or, to put it in another way, less easy to control. The differentiation is more energetic and rapid than is desirable. The whole process of differentiation only lasts some seconds; evidently, then, an error of judgment of only a few seconds may entirely vitiate the result.

728. KAISER'S **Modification of Weigert** (*Neurol. Centralb.*, xii, 1893, No. 11, pp. 364, 368; *Zeit. f. wiss. Mik.*, xi, 2, 1894, p. 249; anterior methods suppressed).—Put the material into liquid of Müller. After two or three days divide it into slices of two to four millimetres in thickness, and put them back into the liquid of Müller for five or six days more. Then put them for eight days into liquid of Marchi (§ 733). Wash, pass through alcohol, and imbed in celloidin. Make sections, and mordant them for five minutes in the following mixture:

Liquor Ferri Sesquichlorati .	1 part.
Aq. Dest.	1 ,,
Spirit. Rect.	3 parts.

Wash them in Weigert's hæmatoxylin, then warm them in a fresh quantity of the same (not to boiling-point) for a few minutes. Wash with water and differentiate in Pal's liquid. Neutralise the oxalic acid by washing in water containing a little ammonia.

729. KULTSCHITZKY (*Anat. Anz.*, 1889, p. 223, and 1890, p. 519; *Zeit. f. wiss. Mik.*, vi, 2, 1889, p. 196, and vii, 3, 1890, p. 367) has given two forms of his well-known process, of which the following is the later:—Specimens are hardened for one or two months in solution of Erlicki, imbedded in celloidin or photoxylin, and cut. Sections are stained for from one to three hours, or as much as twenty-four, in a stain made by adding 1 grm. of hæmatoxylin dissolved in a little alcohol to 100 c.c. of 2 per cent. acetic acid. They are washed out in saturated solution of carbonate of lithium or sodium.

By adding to the carbonate of lithium solution 10 per cent. of a 1 per cent. solution of red prussiate of potash, and decolourising therein for two or three hours or more, a finer differentiation is obtained. After this the sections are well washed in water and mounted in balsam.

730. WOLTERS (*Zeit. f. wiss. Mik*, vii, 4, 1891, p. 466) proceeds as Kultschitzky, except that he stains in a solution kept warm by placing it on the top of a stove kept at 45° C. for twenty-four hours, after which time the sections are dipped in solution of Müller, and differentiated by the method of Pal.

KAES (*ibid.*, viii, 3, 1891, p. 388; *Neurol. Centralb.*, 1891, No. 15) modifies this by staining for as much as two or three days, and performing the differentiation several times over. It appears doubtful whether either of these modifications is an improvement.

731. BERKLEY'S **Rapid Method** (*Neurol. Centralb.*, xi, 9, 1892, p. 270; *Zeit. f. wiss. Mik.*, x, 3, 1893, p. 370).—Slices of tissue of not more than two and a half millimetres in thickness are hardened for twenty-four to thirty hours in mixture of FLEMMING, at a temperature of 25° C. Without washing out, they are brought into absolute alcohol, which is changed twice during the first twenty-four hours. After sufficient hardening they are imbedded in celloidin and cut. After washing in water the sections are put overnight into a saturated solution of acetate of copper (or they may be simply warmed therein to 35° to 40° C. for half an hour). They are then washed, and stained for fifteen to twenty minutes in the fluid given below, warmed to 40° C., allowed to cool, and differentiated for one to three minutes in WEIGERT'S ferricyanide liquid, which may be diluted if desired with one third of water. Water, alcohol, bergamot oil, xylol-balsam.

The stain is made as follows:—Two cubic centimetres of saturated solution of carbonate of lithia are added to 50 c.c. of boiling water, and the solution boiled for two minutes more, when 1½ to 2 c.c. of 10 per cent. solution of hæmatoxylin in absolute alcohol are added.

This method is most suited to fresh material, and does not give good results with tissues that have suffered *post-mortem* changes.

732. Other Modifications or Similar Methods.—FLECHSIG (*Arch. f. Anat. u. Phys.*, Phys. Abth., 1889, p. 537; *Zeit. f. wiss. Mik.*, vii, 1890, p. 71; *Journ. Roy. Mic. Soc.*, 1890, p. 538; BREGLIA, *Zeit.*, vii, 2, 1890,

p. 36; Rossi, *ibid.*, vi, 2, 1889, p. 182; Mercier, *ibid.*, vii, 4, 1891, p. 480; Haug, *ibid.*, vii, 2, 1890, p. 153; Walsem, *ibid.*, xi, 2, 1894, p. 236.

Other Myelin Stains.

733. Marchi's **Method for Degenerate Nerves** (*Rivista sperim. di Fren. e di Med. legale*, 1887, p. 208; taken from the report by Schiefferdecker of a paper by Eijkman, in *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 350).—Nerves are first hardened for a week in solution of Müller, and then put for a few days into a mixture of 2 parts solution of Müller and 1 part 1 per cent. osmic acid solution. The treatment with the chrome salt deprives the medullary sheath of normal fibres of the faculty of impregnating with osmium, whilst the degeneration products in diseased sheaths retain that faculty. In consequence the sheaths in normal nerves acquire a yellow coloration, those of degenerated tracts a black one.

For the study of degenerate nerve-tracts the method of Marchi has an advantage over that of Weigert, in that it gives *positive* images of the degenerated elements, Weigert's process only giving negative ones.

734. Azoulay's **Osmic Acid Method** (*Anat. Anz.*, x, 1, 1894, p. 25).—An application of the osmic acid stain given § 377. (A) Material that has been for several months in liquid of Müller is washed for a couple of days in water, imbedded in celloidin, and sectioned. The sections washed in water are put for five to fifteen minutes into solution of osmic acid of 1 : 500 or 1 : 1000 strength. Rinse with water and put them for two to five minutes into a 5 or 10 per cent. solution of tannin, warming them therein over a flame till vapours are given off, or in a stove at 50° to 55° C. Wash for five minutes in water, double-stain if desired with carmine or eosin, and mount in balsam. Thin sections are necessary to ensure good results. If they should be too thick it will be necessary after staining to differentiate by Pal's process, or by eau de Javelle diluted with 50 vols. of water. (B) Material that has been in an osmic mixture (liquid of Flemming, of Marchi, or of Golgi). Sections as before, then the tannin bath, warming for three to ten minutes, and the rest as before.

735. Osmic Acid (Exner, *Sitzb. k. Akad. Wiss. Wien*, 1881, lxxxiii, 3 Abth.; Bevan Lewis, *The Human Brain*, p. 105).—A small portion of brain, not exceeding a cubic centimetre in size, is placed in ten times its volume of 1 per cent. osmic acid. The solution should be replaced by fresh after two days, a proceeding which may advantageously be repeated at the end of the fourth day. In from five to ten days the piece is usually hardened throughout, and may be washed with water, treated with alcohol, and imbedded. The sections may be treated by a drop of caustic ammonia, which clears up the general mass of the brain substance, leaving medullated fibres black. B. Lewis says that this method exhibits a wealth of structure which no other method displays. The sections may be mounted in soluble glass. The chief value of this method is for tracing the course of medullated fibres.

BELLONCI (*Arch. Ital. de Biol.*, vi, p. 405) employed this method in his researches on the optic nerve of mammalia. He used an osmic acid solution of 0·5 to 1 per cent., hardened for only fourteen to twenty hours, made sections, and treated them for three or four hours with 80 per cent. alcohol, and then with ammonia.

736. The methods of PALADINO (next section) and ZIEHEN (§ 780) are also more or less specific myelin stains.

b. Myelin-and-axis-cylinder Stains.

737. PALADINO'S **Iodide of Palladium Method** (*Rendic. R. Accad. Scienze Fis. e Mat.*, Napoli, iv, 1890, p. 14, and 1891 [1892], p. 227; *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 237, and ix, 2, 1892, p. 238; *Journ Roy. Mic. Soc.*, 1890, p. 817, and 1892, p. 439).—Pieces of material hardened in bichromate, chromic acid, or corrosive sublimate, and not more than 5 to 8 mm. in thickness, are put for two days into a large quantity (at least 150 to 200 c.c. for each piece) of 0·1 per cent. solution of chloride of palladium (which may be made as directed in § 66). They are next put for twenty-four hours into a 1 per cent. solution of iodide of potassium (the later paper quoted says the iodide solution should be of 4 : 100 strength, and that a relatively small volume of it should be taken; otherwise the iodide of palladium, which is rapidly formed in the tissues, may be again extracted by the liquid: small pieces of tissue should not remain in it for more than one or two hours). Dehydrate; imbed, if necessary, in paraffin by the chloroform method; mount in balsam.

A brown stain, being both a myelin stain and an axis-cylinder and cell-process stain, and applicable both to central and to peripheral nervous structures. It is very well spoken of.

738. SAHLI (*Zeit. f. wiss. Mik.*, 1885, p. 1) gives the following method: —Sections of tissue hardened in bichromate to the degree required for

Weigert's hæmatoxylin process are washed for not more than five or ten minutes in water, and stained for several hours, until they are of a dark blue colour, in concentrated aqueous solution of methylen blue. They are then rinsed with water, and stained for five minutes in saturated aqueous solution of Säurefuchsin. If now they be rinsed with alcohol and brought into a liberal quantity of water, the stain becomes differentiated, axis-cylinders being shown coloured red and the myelin sheaths blue. If, instead of rinsing with pure alcohol, alcohol containing from 0·1 to 1 per cent. of caustic potash be taken, the stain differentiated in water, and the sections cleared with cedar oil and mounted in balsam dissolved in cedar oil, still finer images are obtained. Axis-cylinders are red as before, but the myelin sheaths are blue in some places, red in others. Sahli thinks that this reaction points to some difference of kind in the nerve-tubes that exhibit it.

The same author (l. c., p. 50) also gives a method for obtaining a specific stain of nerve-tubes by means of *methylen blue alone*. Sections of material hardened as before are stained for a few minutes or hours in the following liquid:

Water	40 parts.
Saturated aqueous solution of methylen blue .	24 „
5 per cent. solution of borax	16 „

(Mix, let stand a day, and filter.)

The sections are then washed either in water or alcohol until the grey matter can be clearly distinguished from the white, are cleared with cedar oil, and mounted in balsam. Nerve-tubes are stained blue, ganglion-cells greenish, nuclei of neuroglia blue. Micrococci are stained, if any be present in the tissues. The preparations are not perfectly permanent.

739. *Safranin followed by methylen blue* gives a very special stain of spinal cord. The method is due to ADAMKIEWICS (*Sitzb. k. Akad. Wiss. Wien. Math. Naturw. Kl.*, 1884, p. 245; *Zeit. f. wiss. Mik.*, 1884, p. 587). Sections (of material hardened in liquid of Müller for not less than one month and not more than three) are washed first with water, then in water acidified with a little nitric acid, and stained in concentrated solution of safranin. They are then treated with alcohol and clove oil till no more colour comes away, and are brought back again into water, washed in water acidified with acetic acid, stained in methylen blue, and cleared as before. Myelin ("erythrophilous substance" of Adamkiewics) is red, nuclei of nerves, of neuroglia, and of vessels violet. The erythrophilous substance of pathological nerve-tubes does not take the stain, so that the method is valuable for the study of degenerative changes.

740. NIKIFOROW (*Zeit. f. wiss. Mik.*, v, 3, 1888, p. 338) has a modification of the foregoing method, which consists in impregnating with gold chloride or other metallic salt after the safranin stain.

741. Similar to the methods of ADAMKIEWICS are those of CIAGLINSKI (*Zeit. f. wiss. Mik.*, viii, 1, 1891, p. 19) and of STROEBE (*ibid.*, x, 3, 1893, p. 336), both of them employing safranin followed by anilin blue. For NISSL's Congo-red method, see *Münchener med. Wochenschr.*, 1886, p. 528, or *Zeit. f. wiss. Mik.*, iii, 3, 1886, p. 398.

CHAPTER XXXIII.

NEUROLOGICAL METHODS, AXIS-CYLINDER AND PROTOPLASM STAINS (GOLGI AND OTHERS).

742. Introduction.—There are three chief methods for the study of axis-cylinders and protoplasmic nerve-cell processes, viz. the methylen blue method, the sublimate method of GOLGI, and the bichromate-of-silver method of GOLGI. The methylen blue method may be considered to be a *staining* method in the proper sense of the word, whereas the methods of Golgi are true *impregnation* methods. The methylen blue method having been given in Chap. XVII, it remains to group together here some other subordinate but useful methods that are also stains proper; after which will be given the methods of GOLGI and some other impregnation methods.

c. *Stains Proper.*

743. *Ammonia-carmine* is old-fashioned, but may be used for general views. Beale's formula is a good one, especially where prolonged staining is required. The secret of success lies in staining very slowly in extremely dilute solutions. Bichromate material ought to be brought direct into the stain without passing through alcohol (see § 94).

Picro-carmine has much the same action as ammonia-carmine, but gives a better demonstration of non-nervous elements.

Chromic objects stain very slowly in both these media. Sections may, however, be stained with them in a few minutes if they be put into a watch-glass with the stain, and the whole be kept on a wire net over a water-bath heated to boiling-point (OBERSTEINER).

HENLE (*Handb. d. Nervenlehre*, 1871) gives the following, after MERKEL. In order to do away with the slowness of staining of tissues hardened in chromates, sections should be placed in solution of chloride of palladium (1 in 300 to 1 in 600) till they are of a straw-colour (one or two minutes), rinsed in water, and stained in strong ammonia-carmine. Myelin, yellow; axis-cylinders, nerve-cells, and neuroglia, deep red.

Borax-carmine is chiefly useful when employed for double-staining with indigo-carmine or an anilin blue to follow. I have obtained some superb stains with Seiler's borax-carmine and indigo-carmine process (§ 339). MERKEL's mixture of borax-carmine and indigo-carmine (§ 340) has been strongly recommended by MAX FLESCH (*Zeit. f. wiss. Mik.*, 1884, p. 566, and 1885, p. 349), who says that it gives extremely rich and instructive images.

Alum-carmine (Grenacher's or Csokor's) may be used as a nuclear stain (OBERSTEINER). The stain principally takes effect on non-nervous nuclei.

See also SCHMAUS (*Münch. med. Wochenschr.*, 1891, No. 8; see *Zeit. f. wiss. Mik.*, viii, 2, 1891, p. 230, and *Journ. Roy. Mic. Soc.*, 1892, p. 439); UPSON (*Neurolog. Centralb.*, 1888, p. 319; *Zeit. f. wiss. Mik.*, v, 4, 1888, p. 525); FREEBORN (*Amer. Mon. Mic. Journ.*, 1888, p. 231; *Journ. Roy. Mic. Soc.*, 1889, p. 305).

744. *Anilin blue-black* was first recommended by SANKEY (*Quart. Journ. Mic. Sci.*, 1876, p. 69). He stained in a 0·5 per cent. solution, and, in order to obtain a differential stain, washed out for twenty to thirty minutes in solution of chloral hydrate. BEVAN LEWIS (*Human Brain*, p. 125) considers this to be one of the most valuable stains for nervous centres. He stains sections for an hour in 0·25 per cent. aqueous solution, and clears and mounts (in the case of brain or cord sections); for the cortex of the cerebellum he washes out for twenty to thirty minutes in 2 per cent. chloral solution. SANKEY and STIRLING have also used anilin blue-black in a much weaker solution, which Bevan Lewis does not recommend. VEJAS, however (*Arch. f. Psychiatrie*, xvi, p. 200), obtained good results by staining from eighteen to twenty-four hours in a solution of 1 in 3000.

GIERKE (*Zeit. f. wiss. Mik.*, 1884, p. 379) was not able to obtain good results with anilin black procured in Germany, and finds that the treatment with chloral is injurious to the preservation of the tissues. MARTINOTTI (*ibid.*, p. 478) comes to the same conclusion.

LUYS (*Gaz. méd. de Paris*, 1876, p. 346) greatly recommends the anilin colour known as *Noir Colin*. He stains for three to four minutes in a 0·1 per cent. solution.

JELGERSMA (*Zeit. f. wiss. Mik.*, 1886, p. 39) finds that anilin blue-black gives excellent results *provided that the English preparation of the colour be alone employed.* He makes solutions of 1 : 100, 1 : 800, and 1 : 2000, of which the first stains sections in a quarter of an hour, the second in five hours, the third in twelve hours. The stain takes effect on ganglion-cells and their processes, and on axis-cylinders, but does not demonstrate neuroglia or connective tissue.

SCHMAUS (*Münch. med. Wochenschr.*, No. 8, 1891, p. 147;

Zeit. f. wiss. Mik., viii, 2, 1891, p. 230) recommends English blue-black in 0·25 per cent. solution in 50 per cent. alcohol, with the addition of a little picric acid; sections to be stained for an hour. The addition of picric acid has the advantage of leaving celloidin almost colourless, whilst pure aqueous solutions of blue-black stain it strongly.

745. MARTINOTTI (l. c., 1884, p. 478) finds that *picro-nigrosin* gives very good results, especially for pathological objects. He stains for two or three hours or days in a saturated solution of nigrosin in saturated solution of picric acid in alcohol, and washes out in a mixture of 1 part of formic acid with 2 parts of alcohol until the grey matter appears clearly differentiated from the white to the naked eye.

746. ROSIN (*Neurol. Centralb.*, xii, 1893, p. 1; *Zeit. f. wiss. Mik.*, xii, 1, 1895, p. 77) recommends Ehrlich-Biondi mixture. LINDSAY-JOHNSON (*in litt.*) adds to it about one third of a 20 per cent. (saturated) solution of nigrosin.

747. KAISER (*Zeit. f. wiss. Mik.*, vi, 4, 1889, p. 471) advises, for celloidin sections of spinal cord, naphthylamin brown (obtainable from Grübler). Sections are stained for a few hours in a solution containing 1 part of naphthylamin brown, 200 parts of water, and 100 parts of alcohol, washed with alcohol, cleared with origanum oil, and mounted. Chromophilous ganglion-cells, dark brown; chromophobous cells, light on a dark ground.

748. REHM (*Münch. med. Wochenschr.*, 1892, No. 13; *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 389) gives the following:—Sections (of alcohol-hardened material) stained for five minutes in 1 per cent. ammonia-carmine, and washed out in 70 per cent. alcohol acidified with 1 per cent. of nitric acid; the acid removed by pure alcohol; the sections stained for half a minute in 0·1 per cent. solution of methylen blue, differentiated in alcohol, cleared in origanum oil, and mounted in colophonium. Nuclei and axis-cylinders, red. This stain is said to prove that the nuclei of the ganglion-cells of the rabbit contain only one true nucleolus, instead of two or more, as has been hitherto believed.

749. For a plasma-stain, REHM (l. c.) gives a method modified from NISSL. Sections of alcohol-hardened material are stained for half a minute to a minute in a *hot* 0·1 per cent. solution of methylen blue, washed in 96 per cent. alcohol till no more colour comes away, cleared with origanum oil, and mounted in balsam or benzin-colophonium. Nerve-cells, dark blue; connective-tissue cells lighter, and greenish.

750. To obtain a sharper distinction between nerve-cells and connective-tissue cells, REHM (l. c.) stains as before in the hot methylen-blue solution for not more than half a minute, and washes out as before with 96 per cent. alcohol. The sections are then stained for fifteen to thirty minutes in a 0·1 per cent. solution of fuchsin in 96 per cent. alcohol, washed out for a minute, until no more red colour comes away, in alcohol, cleared in clove oil, and mounted. Nerve-cells blue-red, their nuclei being unstained; nuclei of connective tissue and of vessels, brilliant red.

This distinction is not obtained in embryonic tissues.

Nuclei of connective tissue and vessels may also be brought out by staining for a few minutes in 1 per cent. aqueous solution of eosin, followed by a few minutes in warm 0·1 per cent. aqueous solution of dahlia, dehydration, and mounting (the nuclei blue, all else red). Or 1 per cent. aqueous solution of nigrosin may be taken instead of the eosin, and 0·1 per cent. alcoholic solution of fuchsin (half an hour) instead of the dahlia (nuclei red, all else grey-blue).

751. For MALLORY'S *Phospho-molybdic Acid Hæmatoxylin Stain*, see *Anat. Anz.*, 1891, p. 375; *Zeit. f. wiss. Mik.*, viii, 3, 1891, p. 341; MERCIER, *Les Coupes du Système nerveux Central*, p. 228. SCHIEFFERDECKER and VOBIS find that celloidin sections of material hardened in Müller stain well in it.

752. WOLTERS'S **Chloride of Vanadium** process for axis-cylinder and cell-staining is as follows (*Zeit. f. wiss. Mik.*, vii, 4, 1891, p. 471):

The material (either central or peripheral nervous tissue) is *hardened in liquid of* KULTSCHITZKY, § 53, followed by alcohol, *as there described*. It is imbedded either in celloidin or paraffin, and cut. The sections are mordanted for twenty-four hours in a mixture of 2 parts of 10 per cent. solution of chloride of vanadium and 3 parts of 3 per cent. solution of acetate of aluminium, washed for ten minutes in water, and stained for twenty-four hours in a solution of 2 grammes of hæmatoxylin (dissolved in a little alcohol) in 100 c.c. of 2 per cent. acetic acid. They are washed out until they are of a light blue-red colour (it is not possible to specify exactly the time required) in 80 per cent. alcohol acidulated with 0·5 per cent. of hydrochloric acid. Remove the acid thoroughly by washing with pure alcohol, dehydrate, clear with origanum oil, and mount.

A sharp axis-cylinder stain, myelin being coloured only if the differentiation in the acid alcohol is insufficient.

753. ALT (*Münch. med. Wochenschr.*, 1892, No. 4; *Zeit. f. wiss. Mik.*, ix, 1, 1892, p. 81) stains for a couple of hours in solution of Congo in absolute alcohol, and washes out with pure alcohol. The results are said to be specially adapted to the study of peripheral axis-cylinders. SCHIEFFER-

DECKER, reporting on the method, does not recommend it. SQUIRE (using a 2 per cent. aqueous solution) says it is one of the best stains he has tried.

d. Impregnations.

754. The Methods of GOLGI. There are two methods of GOLGI, viz. the **Corrosive Sublimate Method**, and the **Bichromate and Nitrate of Silver Method**. The corrosive sublimate method will be given later on.

The bichromate and nitrate of silver method has been worked out by GOLGI in *three* forms. These are known as the *slow* process, the *rapid* process, and the *mixed* process.*

The rapid process is the one that is the most in use at the present time for researches into the distribution and relations of axis-cylinders and protoplasmic processes; it may be taken to be the classical method of inquiry into the finer relations of the neurons in hardened tissue. It may be said that it is to the study of hardened tissue that which the methylen blue method is to the study of fresh tissue.

Before proceeding to describe these processes it is desirable to indicate briefly the general characters of the impregnations obtained by them. The preparations have not in the least the appearance of stains, and are even very different in aspect from the impregnations obtained on fresh tissue by the ordinary methods of impregnating with nitrate of silver or chloride of gold. The impregnation is a *partial* one, by which is meant that of all the elements, whether nervous or not, that are present in a preparation, only a limited number are coloured. That is the peculiar quality—not by any means the defect, but rather the advantage—of the method. For if all the elements present were coloured equally with the great intensity with which they take the colour in this method, you would not be able to see the wood for the trees, in fact you would hardly be able to distinguish any detail at all in the preparations. But Golgi's method selects from among the elements present a small number which it stains with a great intensity and very completely, that is to say, throughout a great length, so that they are at the same time very clearly separated from those elements that have remained uncoloured, such as supporting cells and the like, and can be followed out for a great distance.

---

* In a recent text-book, the *Leitfaden* of RAWITZ, the sublimate method is called "the slow method of GOLGI," and the bichromate and silver nitrate method is given under the form of the slow process, and called "the rapid method of GOLGI." That is a very "nice derangement of epitaphs" indeed. RAWITZ further attributes the rapid method to RAMÓN Y CAJAL, which is equally erroneous. Similar confusions are made by MERCIER in his *Coupes du Système Nerveux Central.*

There is no other method which will allow cell-processes to be followed out for such great distances. But the method does not demonstrate at the same time the histological detail of other tissues that may be present in the preparations, and all cytological detail is lost. It is *par excellence* a *special* method.

Nervous tissue is not the only thing that is impregnated in these preparations; indeed, the method has been applied with success to the study of such things as bile-capillaries, gland-ducts, and the like. Both on account of this character, and on account of the capriciousness with which the impregnation takes hold of only certain elements of the preparations, care must be exercised in the interpretation of the images obtained. It has been constantly taught in the chapter devoted to gold impregnations in this book (see § 366) that "the very best gold preparations give images that are only worthy of credence as to what they show, and furnish absolutely no evidence whatever as to the non-existence of anything that they do not show; for you can never be sure that the imbibition of the salt has not capriciously failed, or its reduction capriciously stopped, at any point." This warning applies with at least equal force to Golgi's impregnations. And in their case a further source of error is found in the fact that the method frequently gives precipitation-forms of chromate of silver that simulate dendrites and other structures (see the paper of FRIEDLAENDER in *Zeit. f. wiss. Mik.*, xii, 2, 1895, p. 168, and the plate in the following number). A correspondent writes me that he has "Golgified a potato, and obtained beautiful nerve-fibres," and FRIEDLAENDER'S paper describes similar results obtained with white of egg, &c. Clearly, then, caution is necessary in the interpretation of the images.

It has been said that the method does not give good results with the tissues of invertebrates. It has, however, been applied with success by RAMÓN Y CAJAL to the study of the muscles of insects; by VON LENHOSSÉK to the nerves of *Lumbricus;* by RETZIUS to the retina of Cephalopods; and other anatomists have been successful with various objects.

The details of the method have been considerably modified at the hands of various workers, the most important modification being that of the "double" or "intensified" impregnation of RAMÓN Y CAJAL.

The method has been described at length by GOLGI in the *Archives Italiennes de Biologie,* t. iv, p. 32, *et seq.* The following account is from the paper by GOLGI himself in the *Archives de Biologie,* t. vii, p. 15, *et seq.* The account given in former editions of this work represents an earlier form of the method, and should not be followed.

755. GOLGI'S **Bichromate and Nitrate of Silver Method,** *SLOW Process* (l. c., p. 17).—(*a*) *The hardening.*—This must be done in a bichromate solution. Either pure bichromate of potash may be employed or liquid of Müller (the reaction can be obtained with liquid of Erlicki, but it is not to be recommended). The normal practice is to take bichromate of potash, beginning with a strength of 2 per cent., and changing this frequently for fresh solution of gradually increased strength, 2½, 3, 4, and 5 per cent. The tissue to be operated on should be as fresh as possible; though satisfactory results may be obtained from material taken twenty-four to forty-eight hours after death. It should be in pieces of not more than 1 c.cm. or 1½ c.cm. in size.

The most difficult point of the method consists in *hitting off the exact degree of hardening* in the bichromate that should be allowed before passing to the next stage of the process, the silver bath. The degree of hardening arrived at in any given time depends on so many factors (state of the tissues, temperature, &c.) that it is impossible to formulate rules in this respect. In summer good results may be obtained after fifteen to twenty days, and the material may continue in a favorable state for impregnation up to thirty, forty, or fifty days. In cold weather good results can seldom be obtained under a month; when obtained, the material may continue to give good results up to two, three, and even four months of hardening. The only way to make sure is to pass trial portions of the tissue at intervals into the silver bath, in summer frequently, in winter every eight or ten days, and observe whether the reaction is obtained.

Good results are obtained by injecting the organs with the hardening fluid (2·5 per cent. bichromate).

Stoving at a temperature of 20° to 25° C. is useful for abridging the hardening, but there is risk of over-hardening; and Golgi thinks the results are never quite so delicate as after hardening in the cold.

(*b*) *Impregnation.*—As soon as the pieces of tissue have attained the proper degree of hardening in the bichromate, they are brought into a bath of nitrate of silver. The usual strength of this bath is 0·75 per cent., but it is not necessary to hold rigorously to this strength: 0·50 per cent. may be taken, and even seems to be better for material that has not been quite enough hardened, and solutions of 1 per cent. may also be used and even seem to be better adapted for material that has been slightly over-hardened.

A relatively large quantity of solution should be taken for the bath. For two or three pieces of tissue of 1 c.cm. each, Golgi says that about "half a glassful of solution" should be taken (that distinguished anatomist having evidently forgotten for the moment that glasses vary in capacity from a liqueur-glass to a soda-water tumbler).

The moment the pieces of tissue are put into the silver bath, an abundant yellow precipitate of chromate of silver is formed. This of course weakens the bath *pro tanto*. It is therefore well, before putting the pieces into the final silver bath, to first wash them well in a weaker silver solution, until on being put into a fresh quantity of it no further precipitate is formed. Used solutions will do for this purpose. The final silver bath in general needs no further attention, unless it be that sometimes, in the case of tissues that have taken up a great deal of bichromate of potash, the solution may after six to ten hours become somewhat yellow, in which case it should be changed for fresh.

It is not necessary to keep the preparations in the dark during the impregnation-bath; in winter it is well to keep them in a warm place.

The time necessary for impregnation by the silver salt is from twenty-four to forty-eight hours. The normal time is from twenty-four to thirty hours, forty-eight being quite exceptional. By this is meant that the reaction is not obtained in less time, but tissues may remain in the bath without hurt for days, weeks, or months.

(*c*) *Preservation.*—As soon as a trial has shown that a sufficiently satisfactory impregnation has been obtained, the pieces are brought into alcohol. The alcohol is changed two or three times, or even more, until it remains transparent even after the preparations have been two or three days in it. For in view of good preservation it is necessary that the excess of nitrate of silver should be washed out from them thoroughly.

Sections are now made. They are to be washed very thoroughly in three or four changes of absolute alcohol. They are then cleared, first in creosote, in which they should remain only a few minutes, then in oil of turpentine, in which they should remain for ten to fifteen minutes (they may remain there for days without hurt). They are then mounted in Damar (rather than in balsam), *and without a cover.* Preparations mounted under covers in the usual way always go bad sooner or later, whilst those that are mounted without a cover keep very well, *especially if they be kept in the dark.* GOLGI states that he has a large number that have kept without change for nine years.

The order in which the elements of tissues impregnate is —first, axis cylinders, then ganglion cells, and, lastly, neuroglia cells.

756. GOLGI'S **Bichromate and Nitrate of Silver Method,** *RAPID Process* (*op. cit.*, p. 33).—Small pieces of very fresh tissue are thrown into the following mixture:

Bichromate solution of 2 to 2·5 per cent. strength, 8 parts.
Osmic acid of 1 per cent. strength . . . 2 ,,

The hardening being much more rapid than with the slow process, the tissues will begin to be in a fit state for taking the silver impregnation from the second or third day; in the next following days they will be in a still more favorable state, but the favorable moment does not last long, the faculty of impregnation soon declines, and is generally quite lost by the tenth or twelfth day.

The silver impregnation is conducted exactly as in the slow process, and sections are prepared and mounted in the same manner.

There is this difference, that the impregnated material cannot be preserved for any length of time in alcohol, but must not remain for more than two days in it. But it may be kept until wanted for sectioning in the silver solution.

This process has the advantage of great rapidity, and of sureness and delicacy of result, and is the one that has found the most favour with other workers. But for methodical study of any given part of the nervous system, GOLGI himself prefers the following.

757. GOLGI'S **Bichromate and Nitrate of Silver Method,** *MIXED Process* (*op. cit.*, p. 34).—Fresh pieces of tissue are put for periods varying from two to twenty-five or thirty days into the usual bichromate solution. Every two or three or four days some of them are passed on into the osmio-bichromate mixture of the rapid process, hardened therein for from three or four to eight or ten days, and finally impregnated with silver, and subsequently treated exactly as in the rapid process.

The reasons for which Golgi prefers this process are—the certainty of obtaining samples of the reaction in many stages of intensity, if a sufficient number of pieces of tissue have been operated on; the advantage of having at one's disposition a notable time—some twenty-five days—during which the tissues are in a fit state for taking the silver, and the possibility of greatly hastening the process whenever desired by simply bringing the pieces over at once into the osmic mixture; lastly, a still greater delicacy of result, especially remarkable in the demonstration of the "functional" or nervous process of nerve-cells.

758. Critique of GOLGI'S **Method.**—The above-described methods have been found extremely valuable in the most various departments of nervous anatomy. They have given brilliant results in the study of peripheral nerves and their origins or terminations, and in the study of the relations of fibres and cells in the central nervous system. It has been found, at the same time, that they have the defect of considerable uncertainty in the production of the desired reaction, and in the preservation of the stain. These defects have given rise to a most elaborate discussion, which unhappily has not as yet led to very satisfactory results.

Golgi's method is apparently (but this is by no means certain) a double decomposition method, based on the combination of the bichromate of potassium in the tissues with the silver nitrate, and formation of a brown precipitate of bichromate of silver ($K_2Cr_2O_7 + 2AgNO_3 = Ag_2Cr_2O_7 + 2KNO$; the precipitate is brown by reflected light, but appears black by transmitted light). The problem is to preserve this precipitate in the tissues free from chemical or molecular change. And the problem is not an easy one; without special precautions the preparations will not resist the processes necessary for imbedding, will not always resist those necessary for merely mounting in balsam, and even then may easily "go bad" after they have been mounted for a short time.

Modifications concerning the Impregnation of the Tissues.

759. Ramón y Cajal, who has done a great deal of important work by Golgi's method, has always used the *rapid* process. For the times and strengths used by him in his researches on the *cerebral cortex* of mammals, see his paper in *La Cellule*, vii, 1891, p. 125, or *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 239; also *Journ. Roy. Mic. Soc.*, 1892, p. 154. He found it useful to adopt Sehrwald's gelatin process (*infra*, section 766) for avoidance of peripheral precipitates. He prefers not to adopt Greppin's treatment with hydrobromic acid, nor Obregia's treatment with gold chloride, finding that although they serve to render the preparations permanent, they obscure the finer relations of fibres.

For embryos of the *fowl* he employs the same process; see his paper in *Anat. Anz.*, v, 1890, p. 85, or *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 235.

760. Ramón y Cajal's **Double-Impregnation Process.**—In a paper on the structure and relations of the *sympathetic ganglia* (which I have not seen, and quote from *Zeit. f. wiss. Mik.*, l. c.) Ramón y Cajal describes a process of "intensified" or "double" impregnation. After hardening for three days (embryos of fowl) in the osmium-bichromate mixture, the preparations are put for thirty-six hours into nitrate of silver solution (0·5 to 0·75 per cent.) They are then brought back into the same osmium-bichromate mixture, or into a weaker one containing only 2 parts of osmic acid solution to 20 of the bichromate. After treatment with this they are washed quickly with distilled water, and put for a second time into the silver solution for thirty-six to forty-eight hours. It is important to hit off the proper duration of the first impregnation in the bichromate. If it has been too long (four days) or too short (one day), the second impregnation will not succeed. In this case a third impregnation must be resorted to, the objects being again treated with the weak osmium-bichromate mixture, and afterwards again with the silver solution.

This modification of the original process is, perhaps, the most important that has hitherto been made.

761. Kallius (*Anat. Hefte*, x, 1894, p. 527; *Zeit. f. wiss. Mik.*, xi, 2, 1894, p. 154) states that he has often found it advantageous to employ bichromate of ammonia or of soda instead of the bichromate of potash, and to perform all the reactions in the dark. Preparations made by the ammonia or soda salt rarely require a double impregnation.

762. Smirnow (*Intern. Monattsschr.*, x, 2, 1893, p. 241; *Zeit. f. wiss. Mik.*, x, 2, 1893, p. 254), for the study of nerve endings in the human skin, performs the hardening in Altmann's mixture (equal parts of 5 per cent. bichromate of potash and 2 per cent. osmic acid), leaving the pieces therein for 5 to 10 days, and impregnating with 1 per cent. solution of nitrate of silver.

For nerve endings in the skin of the earthworm, he hardens (*Anat. Anz.*, ix, 9, 1894, p. 571) in a mixture of equal parts of 5 per cent. bichromate and 1 per cent. osmic acid, for from 5 to 28 days.

763. Reviving Over-Hardened Tissues.—Tissues that have been too long (three to four weeks) in the osmium-bichromate mixture will no longer take on the silver impregnation, as has been explained above. They can, however, be revivified, and made to impregnate, in the following manner, due to Golgi, and published by Sacerdotti (*Intern. Monatsschr.*, xi, 1894, 6, p. 326; *Zeit. f. wiss. Mik.*, xi, 3, 1894, p. 389). They are washed in a half-saturated solution of acetate of copper until they no longer give a precipitate, and are then put back again for five or six days into the osmium-bichromate mixture. Sections of the impregnated material give remarkably fine images, and will bear mounting in thickened oil of cedar under a cover.

764. Boehm, and afterwards Oppel (*Anat. Anz.*, v, 1890, p. 143, and vi, 1891, p. 165; *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 222, and viii, 2, 1891, p. 224) have modified the hardening part of the process by taking instead of bichromate of potash (slow process) the one, an 0·5 per cent. solution of chromic acid (forty-eight hours), the other, an 0·5 per cent. solution of neutral chromate of potash (twenty-four hours, the pieces having been previously hardened in alcohol). This is for liver.

765. Formaldehyde Mixtures.—Strong (*Anat. Anz.*, x, 15, 1895, p. 494) states that formaldehyde can with advantage be substituted for the osmic acid in the osmio-bichromic mixture of Golgi's impregnation process. He adds from 2·5 to 5 per cent. of "formaline" to the 3·5 to 5 per cent. bichromate solution.

The advantage of using formaldehyde instead of osmic acid is stated by Strong to be that the stage of hardening favorable for impregnation lasts longer; in other words, the formaldehyde-bichromate does not over-harden.

Durig (*ibid.*, p. 659) makes the same recommendation. He obtained the best results by means of 3 per cent. bichromate solutions containing 4 to 6 per cent. of formaldehyde (if I understand rightly). Durig hardens therein for three days, then performs double impregnation by Ramón y Cajal's process.

Fish (*Proc. Amer. Mic. Soc.*, xvii, 1895, p. 319) has also obtained good results with the following mixtures for the Golgi process:

Formalin	2 c.c.
3 per cent. bichromate	100 ,,

leaving the tissues three days in this liquid and three days in the silver nitrate (¾ per cent.).

Or, with advantage:

Liquid of Müller	100 c.c.
10 per cent. formalin	2 ,,
1 per cent. osmic acid	1 ,,

The formalin and bichromate mixtures should be kept in the dark. It is well only to make them up at the instant of using them.

Kopsch (*Anat. Anz.*, xi, 1896, p. 727) states that he has obtained good impregnations with a mixture of 4 parts of 3·5 per cent. bichromate solution, and one of commercial formaldehyde solution. He considers the results more certain than with the osmic acid mixture.

VAN GEHUCHTEN (*in litt.*) has tried the substitution of formaldehyde for the osmic acid in the Golgi process, and has not obtained good results.

766. SEHRWALD'S **Gelatin Process** (*Zeit. f. wiss. Mik.*, vi, 4, 1889, p. 456).

One of the annoyances of Golgi's process is that it frequently gives rise to the formation at the surface of the preparations of voluminous precipitates that are destructive of the clearness of the images. SEHRWALD finds that this evil can be avoided by putting the tissues into gelatin solution before bringing them into the silver-bath. A 10 per cent. solution of gelatin in water may be made. The tissues are imbedded in this in a paper imbedding box with the aid of a little heat (the gelatin melting at a sufficiently low temperature), and are brought therein into the silver-bath. After the silvering the gelatin is removed by warm water saturated with chromate of silver. MARTINOTTI wraps the tissues simply in blotting-paper.

Modifications concerning the Preservation of the Preparations.

767. SEHRWALD, in an elaborate paper (*Zeit. f. wiss. Mik.*, vi, 4, 1889, p. 443), develops the theory that the deterioration of the preparations is due to solution of the precipitate in the reagents employed in the ulterior processes of preparation—in water, in chlorides (which may be present in alcohol), in the xylol, in the paraffin, in the very balsam itself. He made elaborate attempts (fruitless, but instructive) to turn the difficulty by transforming the soluble chromic salt of silver into some insoluble compound. He tried to replace the acid of the salt; he tried to replace the metal of the salt; he tried to make a sulphide of it; he tried to reduce it to the metallic state with photographic developing solutions (and had some success with hydroquinone, but does not recommend the process on account of its giving rise to deceptive precipitates that simulate organised elements). None of these experiments gave the desired result; and the upshot of the paper is that it is better not to attempt to modify the state of the precipitate, but rather so to modify the preparation liquids as to reduce their solvent action on the precipitate. This may be done by supersaturating them with bichromate of silver before using. The powdered salt should be added to each of them in excess, including the clearing media, the medium used for fixing the sections to the slide, the paraffin itself, the solvent used for removing it, and the balsam, and made to dissolve by the aid of heat. By this means thin sections may be prepared and mounted in balsam without injury to the stain. Sehrwald does not say how long they will keep.

768. SAMASSA (*op. cit.*, vii, 1, 1890, p. 26) points out that bichromate of silver is not soluble either in absolute alcohol, toluol, xylol, paraffin, or Canada balsam, nor otherwise chemically affected by them; and that therefore Sehrwald's explanation is untenable. He thinks that the true explanation of the deterioration of the preparations is that the precipitate forming the impregnation is little by little floated away from the tissues by the mechanical force of the diffusion currents set up on the passage of the preparations through the different reagents, and particularly those long-continued ones caused by the slow drying of balsam under a cover-glass. He recommends, therefore, simply that the preparations be *preserved without a cover*, as directed by GOLGI.

769. FICK (*Zeit. f. wiss. Mik.*, viii, 2, 1891, p. 168) does not admit Samassa's theory of the deterioration of sections through diffusion currents (*supra*). He points out that bichromate of silver is soluble in water, especially with the aid of heat; and after an elaborate series of experiments, concludes that the water of the reagents or damp confined by the cover-glass is the cause of the ruin of the preparations. Watery fluids should be avoided, and sections should be mounted without a cover, or on a cover raised free of contact with the slide by means of wax feet, or the like. Or sections mounted without a cover may be later on provided with one if the balsam be first rendered perfectly anhydrous by careful heating.

This last method is also recommended by HUBER (*Anat. Anz.*, vii, 1892, p. 587; *Journ. Roy. Mic. Soc.*, 1892, p. 707; *Zeit. f. wiss. Mik.*, ix, 4, 1893, p. 479). The reader will of course understand that the balsam is to be heated on the slide, with the section in it, until it immediately sets hard on cooling.

770. Greppin (*Arch. f. Anat. u. Entw.*, Anat. Abth., 1889, Supp., p. 55; *Zeit. f. wiss. Mik.*, vii, 1, 1890, p. 66) finds that by means of hydrobromic acid, suggested by Neumann, preparations made by the slow method may be rendered sufficiently resistent to bear mounting under a cover. After silvering, sections are made with a freezing microtome and treated for thirty to forty seconds with 10 per cent. solution of hydrobromic acid, and may then be well washed in several changes of water and mounted in the usual way. If they be cleared in clove oil and exposed therein to sunlight for ten or fifteen minutes, they will take on a violet tone, and details will be more strongly brought out. It is sometimes well to treat them, after the 10 per cent. acid, with 40 per cent. acid for twenty to thirty seconds. They may also be treated by Pal's modification of Weigert's hæmatoxylin process.

771. Obregia (*Virchow's Archiv*, cxxii, 1890, p. 387; *Zeit. f. wiss. Mik.*, viii, 1, 1891, p. 97; *Journ. Roy. Mic. Soc.*, 1891, pp. 536, 830; *Amer. Mon. Micr. Journ.*, 1891, p. 210) gives the following. Sections of silvered material are made, either without imbedding or after imbedding either in paraffin or celloidin, care being taken in either case not to use alcohol of a lower grade than 94 or 95 per cent. They are brought from absolute alcohol into a mixture of eight to ten drops of 1 per cent. solution of gold chloride with 10 c.c. of absolute alcohol, which should be prepared half an hour beforehand and exposed to diffused light until the sections are placed in it, when it should be put into the dark. After fifteen to thirty minutes therein, according to their thickness, the sections are quickly washed in 50 per cent. alcohol, then in water, then treated for five or ten minutes with 10 per cent. solution of hyposulphite of soda. They are lastly washed well with water, and may then be mounted at once in balsam under a cover, or if desired may be previously stained with carmine or hæmatoxylin, or Pal's modification of Weigert's process, or the like.

Obregia thinks that the reason why Sehrwald did not succeed in substituting gold for the silver in his preparations (vide *supra*) is that he took the gold salt in aqueous solution.

This method is also applicable to material treated by Golgi's sublimate process, § 774.

772. Sala (*Zeit. f. wiss. Zool.*, lii, 1, 1891, p. 18; *Zeit. f.*

wiss. Mik., viii, 3, p. 389), in a paper written in Golgi's laboratory, finds Greppin's hydrobromic acid variation not merely useless, but hurtful. And he thinks that Sehrwald's process for imbedding the material in paraffin with the object of getting very thin sections is a mistake. The chief quality of Golgi's process is that it admits of the following of nerve-cell processes for a *very great distance*. Evidently this cannot be done with very *thin* sections. It is better simply to wash the preparations taken from the silver-bath with water, fix them to a cork with gum, put the whole into alcohol for a few hours to harden the gum, and cut with a microtome without imbedding.

773. Kallius (*Anat. Hefte*, ii, 1892, p. 269; *Zeit. f. wiss. Mik.*, ix, 4, 1893, p. 477) has worked out the following process. Take 20 c.c. commercial hydroquinone developing solution and 230 c.c. distilled water (the hydroquinone solution may be made up with 5 grms. hydroquinone, 40 grms. sodium sulphite, 75 grms. carbonate of potassium, and 250 grms. distilled water). At the instant of using, further dilute the solution with one third to one half its volume of absolute alcohol, and put the sections into it for several minutes; they become dark grey to black. In order to ascertain whether reduction is complete, throw a section into a solution of hyposulphite of soda (about 10 parts to 50 of water): chromate of silver will quickly dissolve, whilst metallic silver will not be attacked. As soon as reduction is complete the sections are put for ten to fifteen minutes into 70 per cent. alcohol, then brought for five minutes into the above-given solution of hyposulphite of soda, and thence into a large quantity of distilled water, where they should remain for twenty-four hours or more. Lastly, dehydrate in the usual way, and mount under a cover. All the details which in the undeveloped preparations were brown are now black on a light ground. After-staining with carmine, &c., may be employed. Other developers were tried, and gave satisfactory reductions, but they caused a red or brownish discoloration of the preparations.

774. Golgi's **Bichromate and Sublimate Method** (*Archivio per le Scienze Mediche*, 1878, p. 3; *Archives italiennes de Biologie*, t. iv, p. 32; *Arch. de Biol.*, t. vii, p. 35).—This method, which may be said to be in principle identical with the bichromate of potash and silver nitrate method of the author, consists, like the latter, of two processes: 1, hardening in bichromate; 2, treatment with bichloride of mercury.

For hardening, use either a solution progressively raised in concentration from 1 per cent. to 2½ per cent., or Müller's solution. It is best to take small pieces of tissue (not more than 1 to 2 c.c.), large quantities of liquid, and change the latter frequently, so as to have it always clear. But the reaction can be obtained with much larger pieces, even entire hemispheres. In this case the brain should at first be treated by repeated injections of the liquid, so as to ensure as rapid a permeation of the interior as possible. Fifteen to twenty days' immersion will suffice, or even six to eight, but twenty to thirty should be preferred, and an immersion of several months is not injurious.

The tissues when hardened are passed direct from the bichromate into the bichloride of mercury. The solutions of the latter, first employed by Golgi, varied from 0·25 per cent. to 0·50 or 1 per cent.; he now always takes 0·50 per cent. The immersion in the bichloride must be much longer than the immersion in the nitrate of silver bath of that process: for the latter, twenty-four to forty-eight hours suffice; but in the bichloride an immersion of eight to ten days is necessary in order to obtain a complete reaction through the whole thickness of the tissues, or for entire hemispheres two months or more. During the bath the bichromate will diffuse out from the tissues into the bichloride, which must at first be changed every day, and later on as often as it becomes yellow. At the end of the reaction the preparations will be found decolourised, and offering the aspect of fresh tissue. They may be left in the bichloride for any time.

In *Rendiconti R. Ist. Lombardo di Sci. Milano*, 2, xxiv, 1891, pp. 594, 656 (see *Zeit. f. wiss. Mik.*, viii, 3, 1891, p. 388) Golgi says that for the study of the diffuse nervous reticulum of the central nervous system the best results are obtained by keeping the preparations in 1 per cent. sublimate for a very long time, two years being not too much in some cases.

The reaction may be said to have begun by the time the tissues are nearly decolourised. From that time onwards sections may be made day by day and examined, and those which it is desired to preserve may be mounted.

Before mounting, the sections that have been cut must be repeatedly washed with water (if it be wished to mount them permanently), otherwise they will be spoilt by the formation of a black precipitate. In the last place quoted Golgi says that after washing they may be toned by putting them for a few minutes into a photographic fixing-and-toning bath, after which it is well to wash them again, and stain them with some acid carmine solution. Mount in balsam or glycerin; the latter seems the better preservative medium.

The result of this process is not a true stain, but an "apparently black reaction," the tissues appearing black by transmitted light, *white* by reflected light. Golgi thinks that there is formed in the tissue elements a precipitate of some substance that renders them *opaque*. The elements acted on are (1) the ganglion-cells, with all their processes and ramifications of the processes. These are made more evident than by any other process except the bichromate and silver-nitrate process. An advantage of the mercury process is that it demonstrates nuclei, which is not the case with the silver process. (2) Connective-tissue corpuscles in their characteristic radiate form. But the reaction in this case is far less precise and complete than that obtained by the silver process. (3) The blood-vessels, and particularly their muscular fibre cells.

The method gives *good* results only with the cortex of the cerebral convolutions, hardly any results at all with the spinal cord, and very scanty results with the cerebellum. And, on the whole, the method shows nothing more than can be demonstrated by the silver-nitrate method, but it is superior to it as regards two points: the reaction can always be obtained with perfect *certainty* in a certain time, and the preparations can be perfectly preserved by the usual methods.

But Golgi holds (*Arch. de Biol.*, t. vii, p. 41), that the method deserves an honorable place amongst neurological methods, by the side of the silver-nitrate methods.

See also FLATAU, in *Arch. f. mik. Anat.*, xlv, 1895, p. 158; *Zeit. f. wiss. Mik.*, xii, 2, 1895, p. 257.

The method is recommended by BLOCHMANN (*Biol. Centralbl.*, xv, 1895, p. 14; *Zeit. f. wiss. Mik.*, xii, 2, 1895, p. 226) for the nervous system of Cestodes.

Modifications of Golgi's Bichromate and Sublimate Method.

774a. TAL (*Gazz. degli Ospitali*, 1886, No. 68) finds that if sections made by this process be treated with solution of sodium sulphide, a much darker stain is obtained. Sections may then advantageously be double-stained with Magdala red.

Golgi's method may be combined with Weigert's nerve stain (see PAL, *Wien. med. Jahrb.*, 1886; *Zeit. f. wiss. Mik.*, v, 1, 1887, p. 93).

775. Cox (*Arch. f. mik. Anat.*, xxxvii, 1891, p. 16; *Journ. Roy. Mic. Soc.*, 1891, p. 420) finds the sublimate and bichromate may be used *together*, and give a uniform impregnation. He used a fluid consisting of 20 parts 5 per cent. bichromate, 20 parts 5 per cent. sublimate, 16 parts 5 per cent. simple chromate of potash, and 30 to 40 parts of water. The mixture should be as little acid as possible. The duration of the impregnation is from two to three months. There is considerable difficulty in preserving sections, which must be made with a freezing microtome, alcohol being avoided, treated with solution of sodium carbonate, and mounted without a cover.

776. Magini (*Boll. Accad. Med. di Roma*, 1886; *Zeit. f. wiss. Mik.*, 1888, p. 87) recommends a development of Golgi's process in which zinc chloride is used in place of sublimate. Portions of tissue of 2 to 3 cm. cube are hardened for at least two or three months in Müller's solution. They are well washed with distilled water, and brought into a 0·1 to 1 per cent. solution of chloride of zinc. This is changed for fresh every day for seven to ten days (until it does not become yellower than bichromate solution). Sections are then made, washed quickly with alcohol, imperfectly cleared with kreasote, and mounted in dammar. This process is said to demonstrate better than Golgi's the finer structure of ganglion-cells and their processes.

777. Flechsig's modifications, see *Arch. f. Anat. u. Phys.*, Physiol. Abth., 1889, p. 537; *Zeit. f. wiss. Mik.*, vii, 1, 1890, p. 71.

778. Monti (*Atti d. R. Accad. dei Lincei Roma*, Rendic., v, 1889, 1 sem., p. 705; *Zeit. f. wiss. Mik.*, vii, 1, 1890, p. 72) describes a brown impregnation brought about by the combined action of bichromate of potash and sulphate of copper. The method does not appear to have been yet brought to a practical form.

779. Ziehen's *gold and sublimate method* (*Neurol. Centralb.*, x, 1891, No. 3, p. 65; *Zeit. f. wiss. Mik.*, viii, 3, 1891, p. 385). Ziehen gives the following derivative of Golgi's sublimate method as an improvement on it:—Small pieces of fresh material are thrown into a large quantity of a mixture of 1 per cent. sublimate solution and 1 per cent. chloride of gold solution in equal parts. They remain therein for at least three weeks, preferably for several months (up to five), by which time they will have become of a metallic red-brown colour. They are gummed on cork and sectioned without imbedding. The sections are treated either with Lugol's solution (§ 73) diluted with four volumes of water, or with dilute tincture of iodine, until duly differentiated, which will require more or less time according to the thickness of the sections. They are then washed and mounted in balsam. The result is a bluish-grey impregnation; both medullated and non-medullated nerve-fibres are stained, also nerve- and glia-cells and their processes.

Other Methods.

780. WEIGERT's **Specific Neuroglia Stain** (WEIGERT, *Beitr. zur Kenntniss der normalen menschlichen Neuroglia,* Frankfurt-a-M., 1895; quoted from *Neurol. Centralbl.,* 1895, xxiii, p. 1146).—Pieces of tissue of not more than half a centimetre in thickness are put for at least four days into "10 per cent. solution of formol." They are then mordanted for four or five days in an incubating stove (or for at least eight days at the temperature of the laboratory) in a solution containing five per cent. of neutral acetate of copper, five per cent. of acetic acid, and two and a half per cent. of chrome alum, in water. (Add the alum to the water, raise to boiling point, and add the acetic acid and the acetate, powdered.)

After the mordanting, the tissues are washed with water, dehydrated, imbedded in celloidin and sectioned. The sections are treated for ten minutes with a one third per cent. solution of permanganate of potash, and well washed in water. They are then treated for two to four hours with a solution of "Chromogen." "Chromogen" is a naphthalin compound prepared by the Hoechst dye manufactory. The solution of "Chromogen" to be used is prepared as follows: five per cent. of "Chromogen" and five per cent. of formic acid are dissolved in water and the solution carefully filtered. To 90 c.c. of the filtrate are added 10 c.c. of ten per cent. solution of sodium sulphite.

After this bath, the sections are put till next day into a saturated (5 per cent.) solution of Chromogen.

They are next carefully washed and submitted to the stain. The stain is a modification of WEIGERT's *fibrin stain.* Instead of saturated aqueous solution of methyl violet, you take a warm-saturated solution in 70 per cent. or 80 per cent. alcohol, decant it after cooling, and add to it 5 per cent. of aqueous solution of oxalic acid. And instead of treating with pure anilin, you take a mixture of equal parts of anilin and xylol. This is afterwards removed from the sections by means of pure xylol, and they are mounted in balsam.

Weigert has concluded from the reactions obtained by this process that neuroglia is not a network of nerve-cell processes, but an intercellular substance of the nature of connective tissue substance.

781. See also Aronson, a galleïn stain, in Mercier's *Coupes du système nerveux Central*, p. 255; Kultschizky, Säurerubin for neuroglia, *Anat. Anzeig.*, viii, 1893, p. 357, or *Zeit. f. wiss. Mik.*, x, 2, 1893, p. 256.

782. Upson's **Methods** (Mercier, in *Zeit. f. wiss. Mik.*, vii, 4, 1891, p. 474; or, in his "*Coupes du système nerveux Central*, p. 234; I pass over the older methods given in *Neurol. Centralb.*, 1888, p. 319; and in *Zeit. f. wiss. Mik.*, 1888, p. 525).

A. **The Gold and Iron Method.**—Material must be very carefully hardened for from four to six months in bichromate of potash (*not* solution of Müller, which does not allow of the same precision of stain). A 1 per cent. solution should be taken at first, and should at first be frequently changed. Only after some weeks should the strength be gradually increased up to 2 or 2·5 per cent. All the hardening should be done in the dark. Over-hardened material is not available. The surface of section of a properly hardened piece of material should show macroscopically no marked difference of colour between the white and the grey matter. If the white substance appear very dark, almost black, and the grey substance whitish grey, good results will probably not be obtained; a medullary stain will probably result, whereas the method ought to give an axis-cylinder and cell-process stain.

After hardening, the specimens are washed with water, and put for two or three days into 50 per cent. alcohol, changed as often as necesssary, and then into 95 per cent. alcohol, in which they should remain until they show a decidedly green coloration (two to four weeks), the alcohol being changed as often as precipitates occur. Sections are made either under water or by the celloidin process (in the former case they should not be allowed to remain in contact with the water longer than is absolutely necessary, and should be put back into alcohol for two or three days before passing to the next stage).

The next stage consists in a bath of from one to two hours' duration in 1 per cent. solution of chloride of gold acidified with 2 per cent. of hydrochloric acid. After this the sections are rinsed with water, and treated for half a minute with a freshly prepared solution of ferricyanide of potassium in 10 per cent. potash solution (a lump of ferricyanide not half so big as a pea to 5 c.c. of the potash solution). They are then washed for half a minute more in pure 10 per cent. potash solution, and after that for some time in distilled water.

The following reducing mixture (which must be freshly made up at the very instant of using it for each section) is now to be prepared:

Acidum Sulfurosum	5 c.c.
Tinctura Iodi (3 per cent. strength) . . .	10—15 drops.
Mix, and add—	
Liquor Ferri Chloridi	1 drop.

You must mix quickly in a graduated glass. The section should now be brought on a piece of filter-paper into a watch-glass, and the reducing mixture should be poured over it evenly at one pour, just as developing solution is poured on to a photographic negative. The section should remain in this bath until it is of a fine rosy red tint, not a second longer, or it will become reddish black and useless. As soon as the rose-red colour appears the section is removed into distilled water. The water is changed once, the section is placed on a slide and brought into absolute alcohol, and after five to fifteen minutes therein into clove oil and mounted in balsam. The sections must be kept in the dark.

(The treatment with ferricyanide of potassium sometimes gives rise to the formation later on of precipitates of Berlin blue in the tissues; and as this treatment is not essential it may be omitted, the sections being simply treated with pure potash solution as directed.)

783. Upson's **Methods.—b. The Gold and Vanadium Method—** Sections (made as before) are placed in the following solution:

1 per cent. chloride of gold solution	5 c.c.
Saturated solution of vanadate of ammonium	10 drops.
Hydrochloric acid	3 „

After two hours therein they are washed with distilled water, and put for half to one minute into a mixture of—

10 per cent. solution of caustic potash	5 c.c.
10 per cent. solution of permanganate of potash	10 drops.
Vanadate of ammonium	a trace.

They are rinsed in distilled water, and treated until they become red, as in method *a*, with the following freshly prepared reducing fluid:

a. 3 per cent. tincture of iodine, to which has been added chloride of tin until of a white or yellowish tint (may be kept in stock)	15 drops.
b. Distilled water	3 c.c.
c. Saturated solution of phosphate of iron in distilled water (the usual pharmaceutical solution)	3—5 drops.
d. Sulphurous acid	3 c.c.

Mix *a*, *b*, and *c*, and add *d*, which will cause a voluminous precipitate, at which instant the mixture is to be poured over the section, or the section brought into it, as in method *a*, the remaining treatment being exactly as there described.

The principle of the two methods is the decoloration of the impregnated elements (especially glia-cells) up to a certain point by means of the potash, or potash and ferricyanide, or the vanadate and permanganate. It is important to take care that this decoloration be not overdone, as if carried too far the axis-cylinders and ganglion-cells will be decolourised.

784. Giacomini's **"Dry" Process for Preserving Brains** (*Arch. per le Scienze Mediche*, 1878, p. 11).—Although this is in intention a *macroscopic* method, it appears worth while, both on account of its thorough success and on account of its suggestiveness, to give a description of it here.

The object is to make "dry" preparations of the encephalon, by which is meant preparations that can be permanently preserved *in the air.* The methods hitherto employed were not successful because they consisted in making preparations that were "dry" in the literal sense of the word—that is, deprived of their natural water; and since brain-substance contains 88 per cent. of water, such preparations could not of course be obtained without so great an amount of shrinkage as to most seriously diminish the scientific value of the result. The principle of Giacomini's method is, on the contrary, to *retain* the natural water of the tissues, or an equivalent for it, by means of impregnation with a hygroscopic substance—glycerin.

The process consists of two divisions:—1, hardening; 2, impregnation with glycerin.

1. For hardening may be used zinc chloride, bichromate of potash, chromic acid, nitric acid, or alcohol.

Chloride of zinc gives the best results. Perfectly fresh brain is put into a saturated aqueous solution of the salt (if there be reason to fear that the tissues are somewhat softened through having been left too long after the death of the subject, it is well first to inject 600 grms. of the solution through the internal carotid arteries). After forty-eight hours' immersion (during which time the floating brain must be turned over three or four times, so that all parts of it may duly come into contact with the liquid) the surface of the brain will have attained a consistency that will allow of the removal of the arachnoid and pia mater. The meninges having been removed the encephalon is put back into the solution for two or three days more, during which time it will be seen that, increasing in specific gravity, it tends towards the bottom of the vessel containing it. When this is seen to happen it must be removed into commercial alcohol, as if allowed to remain longer in the chloride of zinc solution it would take up too much water.

In the alcohol it may remain for an indefinite time, or it may be removed if desired after ten or twelve days. (During the alcohol-bath it must be frequently turned over in order that no malformation may arise from continuance of pressure on the same part.)

It is then removed into glycerin (either pure or with 1 per cent. of carbolic acid). It floats at first, but gradually sinks as the alcohol evaporates. As soon as it has sunk just below the surface it may be removed and exposed to the air.

It is set aside to "evaporate" in a convenient place for a few days. As soon as the surface has become dry, it is varnished with india rubber or (better) with marine glue varnish diluted with a little alcohol. This completes the process.

If it be desired to make dissected preparations, the necessary dissection should be made on removing the encephalon from the alcohol before putting into glycerin.

Bichromate of potash may be used for hardening in solutions gradually increasing in concentration from 2 to 4 per cent. The liquid must be frequently changed; the immersion must be of not less than a month's duration. Six to eight days will suffice for the alcohol-bath, or this may be altogether omitted.

Nitric acid is used in solutions of from 10 to 12 per cent. for twelve to fifteen days. (Encephala float in this liquid, and must therefore be frequently turned over. It is this reagent that gives the *toughest* preparations.)

Concerning the value of the process, Golgi (from whose abstract I take the foregoing account) states that after a series of experiments he is able to affirm that for preservation of the volume, the colour, the finer relations of the parts, and the physiognomy proper to the organ, the process is far superior to any hitherto known. I am able to add that I saw specimens of Giacomini's preparations at the Milan International Exhibition of 1881, and think it would be hard to over-praise their beauty of aspect.

It should be added that histological detail is preserved to a remarkable extent by this process, and that excellent sections may be cut at any time from the hardened brains. And as the preparations take up as little room as possible, there seems no reason why the process should not be generally adopted in medical schools, lunatic asylums, and similar institutions.

The method may also be applied, with most perfect success, to the preservation of small animals entire, such as Batrachia, Reptilia. It is well to inject them with the zinc solution.

Another "dry" method has been given by MAX FLESCH (*Mt. Naturf. Ges. Bern.*, 1887, p. xiii). He hardens in alcohol, and then brings the brains through Calberla's mixture (§ 425) into glycerin containing (1 part to 3000 of) sublimate. He does not appear to varnish his preparations. See *Journ. Roy. Mic. Soc.*, 1888, p. 507.

Retina.

785. Fixation and Hardening.—For section cutting, the retina *of small eyes* is best prepared by fixing the entire unopened bulb with osmium vapour. According to RANVIER (*Traité*, p. 954) you may fix the eye of a triton (without having previously opened the bulb) by exposing it for ten minutes to vapour of osmium. The sclerotic being very thin in this animal, such a duration of exposure is generally sufficient. Then divide it by an equatorial incision, and put the posterior pole for a few hours into one-third alcohol.

Somewhat larger eyes, such as those of the sheep and calf, may be fixed in solutions without being opened. But it is generally the better practice to make an equatorial incision, and free the posterior hemisphere before putting it into the liquid.

The older practice was to use strong solutions of pure osmic acid; but most of the best recent work has been done with chromic mixtures.

LINDSAY JOHNSON (*in litt.*) gets the best results by fixing the globe over the steam of a 2 per cent. osmic acid solution raised nearly, but not quite, to boiling point, for five minutes; or, if the eye be that of an adult, by removing the cornea and lens and injecting three to six drops of a mixture of equal parts of acetic acid and 2 per cent. platinic chloride into the vitreous cavity of the eye. After that the eye is put for twelve hours into the mixture, § 97; it is then washed in running water, and suspended in a large volume of 2·5 per cent. bichromate of potash for two days, then passed gradually through successive alcohols, beginning with 20 per cent., and ending with absolute, taking five days from first to last.

This is the treatment recommended for adult human eyes. The eyes of children, small animals, *e.g.* rabbits, monkeys, and cats, and especially fœtal eyes, will require a much gentler treatment or the retina will shrink into folds and the bulb collapse owing to the small amount of supporting and connective tissue present, and the thinness of the sclerotic. These eyes *should not be opened at all during the fixing stage.* After steaming for two or three minutes over osmic vapour, the bulb should be put entire into mixture of Flemming or Hermann, or the mixture No. 97, for three to six hours, and then into bichromate solution, and only after two or three days should the bulb be opened.

He also finds that eyes are preserved perfectly in a mixture of formaldehyde and platinic chloride, the best proportions being:—Commercial formol or formalin, 4 parts, and 1 per cent. solution of platinic chloride, 30 parts. This mixture hardens slowly, but the retina remains nicely adherent, and the fovea well preserved, provided that the eye has previously been fixed by steaming over a 2 per cent. osmic acid solution raised to nearly boiling point, or solid osmic acid fumes produced by heating the crystals in a test tube. Johnson finds as the result of repeated trials that the preliminary rapid fixing of the bulb over osmic vapour is absolutely essential to prevent the swelling and folding over of the retina at the macula, and to a less extent of the retina generally, nor has he been able to find any reliable substitute for this preliminary treatment. As regards the after-hardening, a number of processes may be employed with good results, Flemming's mixture, Müller's fluid, bichromate solution, formol and platinic chloride, and his mixture (§ 97) being especially recommended.

LEBER (*Münch. med. Wochenschr.*, xli, 30, 1894; cf. *Zeit. f. wiss. Mik.*, xii, 2, 1895, p. 256) confirms Hermann's observation concerning eyes (§ 109). He advises a 4 per cent. solution (formol 1, water 10). After a few days' hardening in this, the eyes may be cut through, it is said, without derangement of the parts. The retina lies flat, and is at least as well preserved as with solution of Müller. The eyes may be passed without hurt direct into successive alcohols; the vitreous will shrink a little, but less rather than more than after solution of Müller. I must be allowed to doubt the correct cytological preservation of the elements by this process.

786. Staining.—RAMÓN Y CAJAL employs the rapid chromate of silver method of Golgi, double impregnation process, as given above, § 760.

KUHNT (*Jen. Zeit. f. Naturw.*, Bd. xxiv, H. 1, 1889, p. 177; *Zeit. f. wiss. Mik.*, vii, 1, 1890, p. 65) employs Pal's modification of Weigert's hæmatoxylin process.

DOGIEL employs the methylen-blue method, as given in §§ 291, 293.

SCHAFFER (*Sitzb. k. Akad. wiss. Wien*, xcix, 1890, 3, p. 110; *Zeit. f. wiss. Mik.*, viii, 2, 1891, p. 227) recommends mordanting sections in 1 per cent. chromic acid for some hours, washing for a *short* time only with water, staining for twenty hours in Kultschitzky's acetic acid hæmatoxylin (§ 729), and differentiating for twelve hours in Weigert's ferricyanide solution.

KRAUSE (l. c., § 788) obtains instructive preparations by treating fresh retina with perchloride of iron or of vanadium in 1 per cent. solution, and then with a 2 per cent. solution of tannic or pyrogallic acid. These reagents only stain the granule layers and the nuclei of the ganglion-cells. The elements of the other layers may then be stained with Säurefuchsin, or some other anilin.

LENNOX (*Arch. f. Ophthalm.*, xxxii, 1; *Zeit. f. wiss. Mik.*, iii, 3, 1886, p. 408; and *Journ. Roy. Mic. Soc.*, 1887, p. 339) has been applying Weigert's hæmatoxylin method to the retina, with some remarkable results.

CUCCATI stains with concentrated aqueous solution of Säurefuchsin, and mounts in balsam.

See also BERNHEIMER, *Sb. k. Akad. wiss. Wien*, 1884; or *Journ. Roy. Mic. Soc.*, 1886, p. 167; and RAMÓN Y CAJAL, *Rev. trim. de Hist. Norm. y Path.*, i, 1888, p. 1; *Anat. Anz.*, 1889, p. 111; *Zeit. f. wiss. Mik.*, v, 3, 1888, p. 373, and vi, 2, 1889, p. 204.

COLUCCI (*Zeit. f. wiss. Mik.*, xii, 1, 1895, p. 87) recommends above everything Paladino's iodide of palladium impregnation, § 737.

787. Sections.—Some workers recommend celloidin; but I see no reason whatever for not employing paraffin. Sections may be mounted in dammar or (FLEMMING) in glycerin.

788. Dissociation Methods.—For maceration preparations you may use weak solutions (0·2 to 0·5 per cent.) of osmic acid for fixation, and then macerate in 0·02 per cent. chromic acid (M. SCHULTZE), or in iodised serum (M. SCHULTZE), or in dilute alcohol (LANDOLT), or in Müller's solution, or (RANVIER, *Traité*, p. 957) in pure water, for two or three days. THIN (*Journ. of Anat.*, 1879, p. 139) obtained very good results by fixing for thirty-six to forty-eight hours in one-third alcohol, or in 25 per cent. alcohol, and then staining and teasing.

SCHIEFFERDECKER macerates fresh retina for several days in the methyl mixture, § 559.

KRAUSE (*Intern. Monatsch. f. Anat. u. Hist.*, 1884, p. 225; *Zeit. f. wiss. Mik.*, 1885, pp. 140, 396) recommends treatment for several days with 10 per cent. chloral hydrate solution. Barrett finds that this process preserves the rods and cones admirably.

Inner Ear.

789. SCHWALBE (*Beitr. z. Phys.*, 1887; *Zeit. f. wiss. Mik.*, iv, 1, 1887, p. 90; *Journ. Roy. Mic. Soc.*, 1887, p. 840).—Fix (cochlea of guinea-pig) for eight to ten hours in "Flemming," wash in water, decalcify (twenty-four hours is enough) in 1 per cent. hydrochloric acid, wash the acid out, dehydrate, and imbed in paraffin.

PRENANT (*Intern. Monatsschr. f. Anat. u. Physiol.*, ix, 1, p. 6; *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 379).—For sections, open the cochlea in solution of Flemming or of Hermann, and fix therein for four to five hours. Avoid decalcification as far as possible, as it is inimical to good preservation of elements; but if necessary, take 1 per cent. palladium chloride. Make paraffin sections and stain with safranin, or with methyl violet B, or with anilin green and orange, or with Renaut's eosin-hæmatoxylin.

Isolation preparations of the stria vascularis may be made by putting a cochlea for a day into 1 per cent. solution of osmic acid, then for four to five days into 0·1 per cent. solution; the stria may then be got away whole.

790. Other Methods.—WALDEYER, Stricker's *Handb.*, p. 958 (decalcification either in 0·001 per cent. palladium chloride containing 10 per cent. of HCl, or in chromic acid of 0·25 to 1 per cent.).

URBAN PRITCHARD (*Journ. Roy. Mic. Soc.*, 1876, p. 211).—Decalcification in 1 per cent. nitric acid.

LAVDOWSKY (*Arch. f. mik. Anat.*, 1876, p. 497).—Fresh tissues (from the cochlea) are treated with 1 per cent. solution of silver nitrate, then washed for ten minutes in water containing a few drops of 0·5 or 1 per cent. osmic acid solution, and mounted in glycerin.

MAX FLESCH (*Arch. f. mik. Anat.*, 1878, p. 300).

TAFANI (*Arch. Ital. de Biol.*, vi, p. 207).

POLITZER, "Die anatomische u. histologische Zergliederung d. menschlichen Gehörorganes," Stuttgart (Enke), 1889 (see *Zeit. f. wiss. Mik.*, vii, 3, 1890, p. 364).—The reviewer (HAUG) here calls attention to the admirable qualities of *phloroglucin* as a decalcification agent for this object (see § 587).

EICHLER (*Abh. d. math-phys. Cl. d. k. Sächsischen Ges. d. Wiss.*, Bd. xviii, 1892, p. 311; *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 380).—Detailed account of manipulations for injection of blood-vessels of the labyrinth.

See also SIEBENMANN, *Die Blutgefässe im Labyrinthe des menschlichen Ohres*, Wiesbaden, Bergmann, 1894; cf. *Zeit. f. wiss. Mik.*, xi, 3, 1894, p. 386.

790a. Methylen Blue for Central Nervous System (SEMI MEYER, *Arch. f. mik. Anat.*, xlvi, 1895, p. 282).—Methylen blue has not hitherto given good results with the *central* nervous system of the higher animals, either by the method of intravenous injection or by that of staining by immersion. MEYER has obtained better results (for the *central* nervous system, not for the peripheral) by means of *subcutaneous* injection. The following points should be observed:—*Large* quantities of solution must be injected, but they may be divided into fractional doses. A young rat will require at least 5 c.c. of 1 per cent. solution; a rabbit of a few weeks about 40 c.c.; a cat of the same size about three times as much. Mice are not good subjects. The total dose should be given in several portions, at intervals of one to several hours. If 20 c.c. of 1 per cent. solution be injected into a young rabbit, and again the same quantity after the lapse of two hours, the subject will generally be found to be dead or dying at the end of two hours more. It is not necessary to wait till death by intoxication has taken place, and after a suitable interval the subject may be killed. It is not necessary to expose the organs to the air for the sake of "oxydising" the stain. They should be thrown direct into the liquid of BETHE, § 294. The liquid ought to be well cooled before use. The preparations should remain in it till the next day.

CHAPTER XXXIV.

SOME OTHER HISTOLOGICAL METHODS.

Connective Tissues.

791. Connective Tissue.—S. MAYER (*Sitzb. k. Akad. Wiss.*, lxxxv, 1882, p. 69) recommends, for staining *fresh* tissue, a solution of 1 gramme of "Violet B" (Bindschedler and Busch, Bâle) in 300 c.c. of 0·5 per cent. salt solution. In this liquid connective-tissue cells stain rapidly and energetically. Elastic fibres and smooth muscle also stain, but of different tints.

For RANVIER's method of artificial œdemata for the study of areolar tissue, see his *Traité*, p. 329.

For FLEMMING's observations on the development of connective-tissue fibrils, see *Festschr. R. Virchow gewidmet*, &c., 1, 1891, p. 215; *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 225.

For PIANESE's very complicated double stain with carmine and picro-nigrosin, see *Journ. Roy. Mic. Soc.*, 1892, p. 292.

FREEBORN (*Amer. Mon. Mic. Journ.*, 1888, p. 231; *Journ. Roy. Mic. Soc.*, 1889, p. 305) recommends (for sections) picro-nigrosin, made by mixing 5 c.c. of 1 per cent. aqueous solution of nigrosin with 45 c.c. of aqueous solution of picric acid. Stain for three to five minutes, wash with water, and mount in balsam. Connective-tissue fibres bright blue, nuclei blackish, all the rest greenish yellow.

792. BENECKE's **Stain for Fibrils.**—BENECKE (*Verh. d. anat. Ges.*, vii Vers., 1893, p. 165; *Zeit. f. wiss. Mik.*, xi, 1, 1894, p. 79) finds that by a slight modification of WEIGERT's **Fibrin Stain** (*post*, § 813) good stains of connective-tissue fibrils and some other elements may be obtained. The modification consists in taking for the decoloration a mixture of anilin with xylol instead of pure anilin, as in WEIGERT's form of the process. BENECKE takes a mixture of two parts of anilin with three of xylol. The process is therefore essentially similar to that of KROMAYER given above, § 665. Connective-tissue fibrils are stained of a deep blue, elastic tissue red or violet, glia-fibres of the same tone as the connective tissue.

793. UNNA's **Specific Stains for Collagen** (*Monatsh. f. prakt. Dermatol.*, xviii, 1894, p. 509; *Zeit. f. wiss. Mik.*, xi, 4, 1894,

p. 518). Unna finds that the method of BENECKE (last §) is unsurpassed for the demonstration of collagen fibrils alone, but prefers the following whenever it is desirable to obtain at the same time good images of the ground substance and of other elements of preparations.

1. **The Orcein Method.**—Sections of alcohol material are stained for five minutes in Grübler's strong solution of polychromatic methylen blue. They are then brought for fifteen minutes into a neutral 1 per cent. solution of orcein in absolute alcohol, rinsed in pure alcohol, cleared in bergamot oil, and mounted. Nuclei blue, collagenous ground-substance dark red, granules of Mastzellen carmine red, protoplasm of Plasmazellen blue.

2. **The Method of Sulphosalts.**—(*a*) Stain for five or ten minutes in an aqueous 2 per cent. solution of Säurefuchsin, rinse, treat for one or two minutes with saturated aqueous solution of picric acid, dehydrate (two minutes) in absolute alcohol saturated with picric acid, rinse with pure alcohol, clear and mount. (*b*) Stain for twenty seconds in aqueous 1 per cent. solution of Wasserblau, rinse, treat for five minutes with neutral aqueous 1 per cent. solution of safranin. Rinse and put into absolute alcohol until the blue colour reappears, clear with bergamot oil and mount. Collagen light blue, nuclei red.

794. Fat.—DEKHUYSEN (see FLEMMING, in *Zeit. f. wiss. Mik.*, 1889, pp. 39, 178) has discovered that fat that has been stained black by treatment with chromo-aceto-osmic acid (*not* with pure osmic acid) is dissolved in the course of a few hours in turpentine. It is dissolved also in xylol, ether, and kreasote. FLEMMING finds that very good demonstration preparations may be made by treating fatty tissue with chromo-aceto-osmic acid, staining with safranin or gentian, and then treating for a few hours with turpentine until all the fat is dissolved. The optical hindrance caused by the high refraction of the fat being thus eliminated, nuclei and cytoplasm may be studied to far greater advantage than in the usual preparations.

795. Granule-cells ("Plasmazellen" and "Mastzellen").—In 1874 there were described by WALDEYER (*Arch. f. mik. Anat.*, Bd. xi) certain special cells existing between the bundles of connective tissue, besides the flat cells, and lymphatic and fat cells. They are large round cells containing large granules; Waldeyer called them *plasma cells* ("Plasmazellen"). Later on, EHRLICH (*ibid.*, xiii, 1877) distinguished in the same tissue and in other places certain cells containing large granules, which have a superficial resemblance to Waldeyer's plasma cells, but which differ from them in staining reaction (*Verhandl. Berl. Physiol. Ges.*, January 17th, 1879; *Reichert. u. Du Bois Reymond's Arch.*, 1879, p. 166). Ehrlich named these *food cells* ("Mastzellen"), intending to express thereby the opinion that these cells are derived from fixed connective-tissue cells by a transformation brought about by exalted nutrition.

If (KORYBUTT-DASZKIEWICZ, *Arch. f. mik. Anat.*, xv, 1878, p. 7) frogs be kept for two months (in summer) without food, then placed in a reservoir of running water and well fed for four weeks, "plasma-cells" will be found in abundance, especially in the neighbourhood of the nerves.

It is a pretty general character of these elements that they take the stain of anilin colours and retain it on treatment with alcohol with greater energy than other tissue cells. And further (at least so far as regards the true Mastzellen of Ehrlich) that in successful preparations they show the nucleus unstained, the general mass of cytoplasm unstained or but slightly coloured, and, in the cytoplasm, the characteristic granules very intensely stained.

The staining reactions of the granules are very similar to those of bacteria. In order to distinguish them from these it may be observed that the stain of anilins is removed from the granules, but not from bacteria, by treatment with a weak solution of carbonate of potassium (SQUIRE, *Methods and Formulæ*, p. 46).

796. EHRLICH'S **Classification of Granules.**—Cell-granules in general have been classified by Ehrlich, according to their respective degrees of affinity for one or the other of the three groups of dyes defined in § 268, into (1) α granulations, or eosinophilous granulations, being such as in a mixture of dyes select the most acid stain there present; (2) the β granulations, or amphophilous granules, being such as in a mixture of basic and acid dyes take up both; (3) the γ or basophilous granules, being such as take up basic dyes only (such are the granules of Mastzellen; (4) the δ granulations, also basophilous; and (5) the ε granulations, or neutrophilous granules, being such as take up neutral anilin dyes, such as methyl blue and "acid" fuchsin [acid fuchsin is, however, a weakly acid dye]. I do not propose to enter minutely into the subject of these reactions, as it is one that cannot profitably be treated apart from the histology of the elements in question, and will confine myself to mentioning a few well-known methods.

Some of these have already been given; viz., the Ehrlich-Biondi mixture, § 306; Ehrlich's C mixture, or acidophilous mixture, § 323, for eosinophilous granulations; and his triacid mixture, § 307, which stains the α, β, and ε granulations.

797. Ehrlich's "**Mastzellen**" (*Arch. f. mik. Anat.*, 1876, p. 263).—The tissues must be first well hardened in strong alcohol (chromic acid and its salts must be avoided). They are then placed for at least twelve hours in a staining fluid composed of—

Absolute alcohol	50 c.c.
Aqua	100 c.c.
Acid. acet. glacial	12½ c.c.

—to which has been added enough dahlia to give an almost saturated solution. After staining, the preparations are transferred to alcohol, which washes out the stain from all but the plasma-cells, and may then be mounted in resin-turpentine solution.

Mucus-cells and fat-cells are also sometimes stained by these solutions.

Other Media.—In a similar way other soluble anilins may be employed (in the form of a fluid containing 7½ per cent. of acetic acid),—primula, iodine violet, methyl violet, purpurin, safranin, fuchsin; of these, methyl violet gives the best results.

798. "**Mastzellen.**"—Schiefferdecker (Schiefferdecker and Kossel's *Gewebelehre*, p. 329) recommends the following, after Orth. A piece of mesentery of a rat is brought into a solution of gentian violet in anilin water (p. 190), which is carefully heated over a flame until vapour begins to be given off and then allowed to remain for a couple of hours (or the heating may be omitted, and the preparation allowed to stand for twenty-four hours). It is then rinsed in water, washed out until almost colourless in hydrochloric acid alcohol, rinsed in water, counterstained (if desired) in carmine, and mounted in balsam. Nuclei red, granules blue.

799. Plasma-cells (Nordmann, *Beitr. z. Kenntniss d. Mastzellen, Inauguraldiss.*, Helmstedt, 1884).—Nordmann finds it useful to employ a solution of vesuvin containing 4 to 5 per cent. of hydrochloric acid. Sections should remain for a few minutes in the solution, and then be dehydrated with absolute alcohol. The paper quoted contains a detailed discussion of the microchemical reactions of granule-cells.

800. Plasma Cells and Mastzellen.—Unna, in his paper, *Zeit. f. wiss. Mik.*, vii, 4, 1892, p. 475, gives the following:

A. *For Plasma Cells.*

Methylen blue	1·0
Caustic potash	0·05
Distilled water	100·0

Add a few drops of this to ten, fifty, or one hundred vols. of anilin water (p. 190) in a watch-glass, and stain (alcohol material, or at most sublimate and alcohol material, not chromic material) for half an hour, several hours, or over night. Dehydrate rapidly in absolute alcohol, differentiate in creosol (details not given), rinse in xylol, and mount in balsam.

B. *General Stain, also bringing out Plasma Cells.*

Methylen blue	1·0
Carbonate of potash	1·0
Distilled water	100·0
Alcohol	20·0

Heat on a water-bath until reduced to 100·0. Use for staining undiluted, or diluted with one vol. of anilin water. Differentiate (details not given) with glycol, styron, or creosol. Mastzellen are not differentiated.

C. *Stain giving Red Mastzellen with Blue Plasma Cells.*

Methylen blue	1·0
Kali Carbon. (natron carbon, ammon. carbon)	1·0
Aq. dest. (Aq. carbolisata, chloroforma)	100·0

Dilute about 100-fold, stain slowly, treat with 70 to 80 per cent. alcohol, differentiate in styron, and bring through bergamot oil or xylol into balsam. In this process the granules of the Mastzellen stain red in consequence of the formation of *methylen red* in the staining bath.

In a paper "Ueber Plasmazellen, insbesondere bei Lupus" (for which see *Monatsh. f. prakt. Dermatol.*, xii, 1891, p. 296, or *Zeit. f. wiss. Mik.*, ix, 1, 1892, p. 92), UNNA gives some directions concerning the process of differentiation with creosol. Creosol only differentiates the stain, it does not dehydrate the sections. Sections should, therefore, first be dried with blotting-paper, and should then be treated with absolute alcohol for a few seconds, or with anilin oil before applying the creosol. The time required for differentiation in the creosol is from a few minutes to a few hours. When the proper stage of differentiation is attained, it should be fixed with xylol and the sections mounted. The method shows Mastzellen of a cherry-red tone.

In a paper, l. c., xiii, 1891, p. 364 (see *Zeit. f. wiss. Mik.*, xi, 1, 1892, p. 89), VAN DER SPEK and UNNA describe some further experiments. Maintaining the method of staining given above under A, they find that besides the differentiating agents given under B, a lengthy series of other reagents are available. In all of them Mastzellen are distinguishable from plasma cells by the reddish coloration of their granules.

See also UNNA, *Monatsh. f. prakt. Dermatol.*, xix, 1894, p. 225 ; *Zeit. f. wiss. Mik.*, xii, 2, 1895. p. 58.

801. Plasma Cells and Mastzellen.—BERGONZINI (*Anat. Anz.*, vi, 1891, pp. 595—600; *Zeit. f. wiss. Mik.*, ix, 1, 1892, p. 95) gives the following:—Mix 1 volume of 0·2 per cent. solution of Säurefuchsin with 2 volumes of a like solution of methyl green, and 2 volumes of a like solution of gold-orange, and filter through cotton wool. Stain alcohol or sublimate sections (after washing in water) for three to four minutes, wash for one or two minutes in water, bring into absolute alcohol for two minutes, clear in bergamot oil or pure creosote, wash in turpentine, and mount in balsam.

One sort of gold-orange precipitates methyl green, and therefore cannot be used in this mixture. Orange G may be used instead (thus giving something very like Ehrlich-Biondi mixture), but in this case the acidophilous granules will stain greyish instead of red or orange. Basophilous granules ought to be green, weakly acidophilous granules red, strongly acidophilous ones orange or brown, nuclei always green. Other details l. c.

802. Plasma Cells.—Jadassohn (*Arch. f. Dermatol. u. Syphilis*, Ergänzungsheft 1, 1892, p. 58; *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 226) recommends staining for not too long in a 1 : 2000 strongly alkaline or borax solution of Thionin, and washing out with acidulated water.

See also von Marschalkò, *Arch. f. Dermatol. u. Syphil.*, xxx, 1895, p. 3; *Zeit. f. wiss. Mik.*, xii, 1, 1895, p. 64.

803. Clasmatocytes.—Ranvier (*C. R. Acad. des Sci.*, 1890) has described under the name of "clasmatocytes" certain cells that occur in the thin connective membranes of vertebrates, and that show a close affinity to Mastzellen, possessing processes that break up and form islands of granules. He demonstrates them as follows :—A piece of suitable membrane (epiploön of mammalia, mesentery of batrachia) is stretched *secundum artem* on a slide, and a few drops of 1 per cent. solution of osmic acid are allowed to fall on to it. After one or two minutes it is washed with water and stained with concentrated aqueous solution of methyl violet 5B diluted with ten parts of distilled water. When sufficiently stained the preparation is covered with a thin glass cover and examined. Glycerin may be added to make the preparation permanent, but does not succeed very well, as it causes the stain to diffuse. Brun's glucose medium (§ 420) would probably be preferable.

804. Elastic Tissue.—Two of the most salient characters of elastic fibres are that they have a great affinity for osmium, staining with much more rapidity than most other tissue-elements, and that they are not changed by caustic soda or potash. A further character is that they have a great affinity for certain anilin dyes, especially Victoria blue.

For a review of the older methods of Balzer, Unna, Lustgarten, and Herxheimer, see the paper by Martinotti in *Zeit. f. wiss. Mik.*, iv, 1, 1887, p. 31.

The method of Lustgarten has been given in § 281. The colour used by him was called "Victoriablau 4B.," and this is probably an important detail.

The method of Martinotti (l.c.) is as follows :—Fix in a chromic liquid, wash, stain for forty-eight hours in strong (5 per cent. Pfitzner's) solution of safranin, wash, dehydrate, clear, and mount in balsam. Elastic fibres are stained an intense black, the rest of the preparation showing the usual characters of a safranin stain.

The staining will be performed quicker if it be done at the temperature of an incubating stove (GRIESBACH, *ibid.*, iv, 1887, p. 442). And FERRIA (*ibid.*, v, 3, 1888, p. 342) says that clearer preparations will be obtained if the sections be left for a long time, say twenty-four hours, in the alcohol, or be treated for a short time with very dilute alcoholic solution of caustic potash. This decolourises more completely the ground of the preparations.

Another safranin method, which seems to have the fault of requiring a too minute attention to details, is that of MIBELLI, see *Mon. zool. italiano*, 1, p. 17, or *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 225 (the report in *Journ. Roy. Mic. Soc.*, 1890, p. 803, is vitiated by a misprint).

805. UNNA'S **Modified Orcein Method** (*Monatsh. f. prakt. Dermatol.*, xix, 1894, p. 397; *Zeit. f. wiss. Mik.*, xii, 2, 1895, p. 240).—The following solution is made :– Grübler's orcein, 1 part; hydrochloric acid, 1 part; absolute alcohol, 100 parts. The sections are put into a porcelain capsule with just enough of the stain to cover them, and the whole is warmed in a stove or over a naked flame to about 30° C. After ten to fifteen minutes the stain becomes quite thick, owing to the evaporation of the alcohol. The sections are then well rinsed in alcohol, cleared and mounted. Elastin dark brown, collagen light brown.

For UNNA'S earlier orcein method, see last edition, or *Monatsh. f. prakt. Dermatol.*, xii, 1891, p. 394 (*Zeit. f. wiss. Mik.*, ix, 1, 1892, p. 94).

See also ZENTHOEFER, in Unna's *Dermatol. Studien*, 1892, or *Zeit. f. wiss. Mik.*, ix, 4, 1893, p. 509; KÖPPEN, *Zeit. f. wiss. Mik.*, vi, 4, 1889, p. 473; and vi, 1, 1890, p. 22, or last edition; BURCI, *Journ. Roy. Mic. Soc.*, 1891, p. 831, and 1892, p. 292, or last edition; HANSEN, Virchow's *Archiv*, cxxxvii, 1894, p. 25; *Zeit. f. wiss. Mik.*, xi, 3, 1894, p. 383; KULTSCHIZKY, *ibid.*, xiii, 1, 1896, p. 74, or the original, *Arch. f. mik. Anat.*, xlvi, 1895, p. 673.

*Bone.**

806. **Bone, Non-decalcified** (RANVIER, *Traité*, p. 297).—Ranvier points out certain precautions that it is necessary to take in the preparation of sections of dry bone. In general, the bones furnished by "naturalists," or procured in anatomical theatres, contain spots of fatty substance that prevent good preparations from being made. Such spots are formed when bones are allowed to dry before being put into water for maceration; when a bone is left to dry the fat of the medullary canals infiltrates its substance as fast as its water evaporates.

---

* For a minutely detailed review (40 pages, with references to 80 memoirs) of the whole subject of the technique of bone, see the paper of SCHAFFER, *Die Methodik der histologischen Untersuchung. des Knochengewebes*, in *Zeit. f. wiss. Mik.*, x, 2, 1893, p. 167.

Bones should be plunged into water as soon as the surrounding soft parts have been removed, and should be divided into lengths with a saw whilst wet. The medulla should then be driven out from the central canal by means of a jet of water; spongy bones should be submitted to hydrotomy. This may be done as follows:—An epiphysis having been removed, together with a small portion of the diaphysis, a piece of caoutchouc tubing is fixed by ligature on to the cut end of the diaphysis, and the free end of the piece of tubing adapted to a tap through which water flows under pressure.

As soon as the bones, whether compact or spongy, have been freed from their medullary substance they are put to macerate. The maceration should be continued for several months, the liquid being changed from time to time. As soon as all the soft parts are perfectly destroyed, the bones may be left to dry. When dry, they should be of an ivory whiteness, and their surfaces exposed by cutting of a uniform dulness.

Thin sections may then be cut with a saw and prepared by rubbing down with pumice-stone. Compact pumice-stone should be taken and cut in the direction of its fibres. The surface should be moistened with water and the section of bone rubbed down on it with the fingers. When both sides of the sections have been rubbed smooth in this way, another pumice-stone may be taken, the section placed between the two, and the rubbing continued. As soon as the section is thin enough to be almost transparent it is polished by rubbing with water (with the fingers) on a Turkey hone or lithographic stone. Spongy bone should be soaked in gum and dried before rubbing down (but see VON KOCH's copal process, *ante*, § 174, and EHRENBAUM's colophonium process, § 175).

The process of WEIL (*Zeit. f. wiss. Mik.*, v, 2, 1888, p. 200), which is intended for bone and teeth, has been given § 176.

RÖSE (*Anat. Anz.*, vii, 1892, pp. 512—519; *Zeit. f. wiss. Mik.*, ix, 4, 1893, p. 506) points out some precautions that it is well to take. He penetrates first with a mixture of cedar oil and xylol, then with pure xylol, and imbeds in solution of dammar in chloroform or xylol. The method can be combined with Golgi's impregnation.

Nealey (*Amer. Mon. Mic. Journ.*, 1884, p. 142; *Journ. Roy. Mic. Soc.*, 1885, p. 348) says that perfectly *fresh* portions of bone or teeth may be ground with emery on a dentist's lathe, and good sections, with the soft parts *in situ*, obtained in half an hour.

White (*Journ. Roy. Mic. Soc.*, 1891, p. 307) recommends the following :—Sections of osseous or dental tissue should be cut or ground down moderately thin, and soaked in ether for twenty-four hours or more. They should then be put for two or three days into a thin solution of collodion stained with fuchsin (made by dissolving the dye in methylated spirit, adding the requisite quantity of ether, then the pyroxylin). The sections are then put into spirit to harden the collodion. After this they are ground down to the requisite thinness between two plates of old ground glass, with water and pumice powder, and mounted, *surface dry*, in stiff balsam or styrax, care being taken to use as little heat as possible. Lacunæ, canaliculi, and dentinal tubuli are found infiltrated by the coloured collodion.

For the method of Matschinsky (*Arch. f. mik. Anat.*, xxxix, 1892, p. 151; *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 353), see last edition.

For a similar infiltration and grinding method of Ruprecht, see *Zeit. f. wiss. Mik.*, xiii, 1, 1896, p. 21, wherein see also quoted (p. 23) a method of Zimmermann.

Hopewell-Smith (*Journ. Brit. Dent. Ass.*, xi, 1890, p. 310; *Journ. Roy. Mic. Soc.*, 1890, p. 529) says that for preparing sections of teeth showing odontoblasts *in situ* the best plan is to take embryonic tissues. A lower jaw of an embryonic kitten or pup may be taken, and hardened in solution of Müller followed by alcohol, then cut with a freezing microtome. The knife cuts quite easily the thin cap of semi-calcified dentine and bone.

807. Vivante (*Intern. Monatssch. f. Anat. u. Phys.*, ix, 1892, p. 394; *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 351) has made out that thin specimens of bone can be successfully treated by Golgi's bichromate of silver process. He places portions of frontal bone of four to six months calves, which are not more than 3 to 4 mm. thick, for eight days in solution of Müller, then in the osmium bichromate mixture, and then in the silver solution. After impregnation the specimens should be decalcified, which may be done by putting them for twenty days into von Ebner's mixture (§ 577), after which they should be well washed with water and brought into solution of carbonate of soda, and finally imbedded in paraffin.

Another method recommended by Vivante is as follows:—Fix for five or six days in solution of Flemming, wash and decalcify with von Ebner's mixture, wash, treat with carbonate of soda, imbed in paraffin, cut, and stain the sections for an hour in 0·2 per cent. solution of quinoleïn blue (§ 321). Wash out in equal parts of alcohol and water followed by pure water, dry the sections in a stove at 40° C. (both alcohol and glycerin must be avoided), clear with bergamot oil, and mount in dammar. Nuclei violet, protoplasm and ground substance different shades of blue.

For UNDERWOOD's gold process for teeth, see p. 257; and for that of LEPKOWSKY, see *Anat. Anz.*, vii, 1892, p. 274; *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 355; or the last edition of this work.

808. Bone, Decalcified (FLEMMING, *Zeit. f. wiss. Mik.*, 1886, p. 47).—Sections of decalcified bone are made with the free hand. They are soaked in water, and brought in a drop of water on to a glass plate, where they are spread out flat. The excess of water is removed with blotting-paper, and the sections are covered with another glass plate, to prevent them from rolling. The whole is brought into a plate and covered with alcohol. After the lapse of half an hour the sections have become fixed in the flat position, and may be brought into absolute alcohol without risk of their rolling. To mount them, wash them with fresh alcohol (which may be followed by ether); lay them again flat on glass, and cover them with a double layer of blotting-paper and a somewhat heavy glass plate, and let them dry for a day in the air or in a stove. When they are dry, put a drop of melted balsam on a slide, and let it spread out flat and cool. Prepare a thin glass cover in the same way, put the section on the prepared slide, cover it with the prepared cover, put on a clip, and warm.

By this process sections can be very expeditiously prepared, which show the lacunar system injected with air in quite as instructive a manner as non-decalcified sections.

KÖLLIKER (*Zeit. f. wiss. Zool.*, xliv, 1886, p. 662) recommends the following process for the demonstration of the fibres of Sharpey in decalcified bone. Sections are treated with concentrated acetic acid until they become transparent, and are then put for one quarter to one minute into a concentrated solution of indigo-carmine, then washed in water and mounted in glycerin or balsam. In successful preparations the fibres of Sharpey appear stained of a pale or dark *red*, the remaining bone-substance blue.

Zachariadès (*Zeit. f. wiss. Mik.*, x, 4, 1893, p. 447) has the following:—Bone is decalcified by means of picric acid, is repeatedly washed until all the acid is removed, then put into alcohol and sectioned. The sections are placed on a slide and treated for a few seconds with 1 per cent. solution of osmic acid. They are then stained, either for twenty-four hours in a weak aqueous solution of quinolein blue, or for a few minutes in a saturated aqueous solution of safranin. They are then treated on a slide with a drop of 40 per cent. solution of caustic potash, the slide being warmed over a flame until the sections spread out flat. The excess of potash is then removed and the sections are carefully washed with water, covered and examined. The safranin preparations may be permanently preserved in glycerin containing a small proportion of safranin. It is said that preparations thus made demonstrate that there exist in fresh bone, at all ages of the subject, ramified cells possessing a membrane, and easily isolated by the action of potash. Their ramifications anastomose with one another and with the ramifications of other cells, the whole forming a protoplasmic network enveloped in a membrane.

809. Stains for Cartilage (and Decalcified Bone).—For an excellent discussion (especially as regards staining) of the methods that have been recently recommended for these objects, see the exhaustive paper of Schaffer in *Zeit. f. wiss. Mik.*, v, 1, 1888, which gives in sufficient detail all the methods in question. The following appear to be the best:—

Schaffer's *safranin method*. This method is due in its principle to Bouma (*Centralb. f. d. med. Wiss.*, 1883, p. 866). I give it in the form to which it has been brought by the careful study of Schaffer (*Zeit. f. wiss. Mik.*, v, 1, 1888, p. 17). Sections of bone decalcified with nitric acid (chromic acid may be used, but the stain will be less brilliantly contrasted) are stained for half an hour to one hour in 0·05 per cent. aqueous solution of safranin, washed with water, put for two or three hours in 0·1 per cent. solution of corrosive sublimate, and examined in glycerin. In order to make permanent preparations, the sections on removal from the sublimate are rinsed with alcohol, pressed on to a slide with filter-paper, cleared *for a long time* in bergamot oil or clove oil, and mounted in xylol balsam.

This is a *double* stain; cartilage, orange; bone, uncoloured (or green in chromic objects); marrow, red.

Bayerl's *method for ossifying cartilage* (*Arch. f. mik. Anat.*, 1885, p. 35), is as follows:—Portions of ossified cartilage are decalcified as directed § 578, cut in paraffin, stained in Merkel's borax-carmine and indigo-carmine mixture, § 340, and mounted in balsam.

Max Flesch (*Zeit. f. wiss. Mik.*, 1885, p. 351) particularly recommends this process for the study of the development of dental tissue.

Aqueous solution of benzoazurin has been commended as a stain for ossifying cartilage by Zschokke, see *Zeit. f. wiss. Mik.*, x, 3, 1893, p. 381.

A process recommended by Baumgarten has been given, § 342.

Moerner (*Skandinavisches Arch. f. Physiol.*, i, 1889, p. 216; *Zeit. f. wiss. Mik.*, vi, 4, 1889, p. 508) gives several stains for tracheal cartilage, chiefly as microchemical tests, for which see the last edition.

See also a critique of these methods by Wolters in *Arch. f. mik. Anat.*, xxxvii, 1891, p. 492; *Zeit. f. wiss. Mik.*, viii, 3, 1891, p. 383; and on the whole subject of cartilage, see Schiefferdecker's *Gewebelehre*, p. 331.

Blood.

810. It might be supposed that for the study of blood it would suffice to prick a finger, place a drop of blood on a slide, cover, and examine it. But this is by no means the case. "*Blut ist ein ganz besonderer Saft,*"* and will not yield up its secrets to such simple wooing. The technique of blood is most elaborate; see, for instance the voluminous work of Hayem, *Du sang et de ses altérations anatomiques*, pp. 1035, with 126 figures, Paris, Masson, 1889 (a report of over twenty pages on this important work is contained in *Zeit. f. wiss. Mik.*, vi, 3, 1889, p. 330, *et seq.*); Löwit, *Sitzb. k. Akad. wiss. Wien*, 3, lxxxviii, 1883; xcii, 1885; xcv, 1887; Ziegler's *Beitr. z. path. Anat.*, x, 1891, p. 214; *Zeit. f. wiss. Mik.*, vi, 1889, pp. 74, 76; viii, 3, 1891, p. 371; *Arch. f. mik. Anat.*, xxxviii, 1891, p. 524; *Zeit. f. Wiss. Mik.*, ix, 2, 1892, p. 233; *Studien zur Physiol. u. Path. d. Blutes u. d. Lymphe*, Jena, Fischer, 1892; Ehrlich, *Zeit. f. klin. Medicin*, i, 1880, 3, p. 558; *Zeit. f. wiss. Mik.*, i, 1884, p. 382, and other papers; Müller, *Sitzb. k. Acad. Wiss. Wien*, xcviii, 3, p. 219; *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 365; Griesbach, *Zeit. f. wiss. Mik.*, vii, 3, 1890, p. 326; and many other investigators.

---

* Goethe's *Faust*, i, 4, line 1387.

It is out of the question for me to attempt to abstract these memoirs in the space at my disposal, so I must confine myself to giving a few methods that may be useful to the general student, referring the specialist to the original papers.

811. Fixing and Preserving Methods.—The time-honoured process of drying drops of blood over a flame gives rise to great deformation of the elements, and should be abandoned as far as possible. It is better to mix the blood at once with some fixing and preserving medium, and study it as a fluid mount. Some examination liquids and stains for fresh blood in the fluid state are given in the next §.

Most recent authors (BIONDI, MOSSO, MAX FLESCH) are agreed that by far the most faithful fixing agent for blood-corpuscles is osmic acid. A drop or two of blood (Biondi recommends two drops exactly) is mixed with 5 c.c. of osmic acid solution, and allowed to remain in it for from one to twenty-four hours. The exact degree of concentration of the osmium solution is a somewhat important point, and must be made out by experiment for each form. As a rule it should be strong, 1 to 2 per cent. According to Biondi, 2 per cent. is best. Fixed specimens may be preserved for use in acetate of potash solution (MAX FLESCH, *Zeit. f. wiss. Mik.*, v, 1, 1888, p. 83).

GRIESBACH also (op. cit., p. 328) prefers osmic acid, not only as being a first-rate fixing agent, but because it can be combined with certain stains without decomposing them. He mentions methyl green, methyl violet, crystal violet, safranin, eosin, Säurefuchsin, rhodamin, and iodine in potassic iodide.

ROSSI (*Zeit. f. wiss. Mik.*, vi, 4, 1889, p. 475) advises a mixture of equal parts of 1 per cent. osmic acid, water, and strong solution of methyl green, permanent mounts being made by means of glycerin cautiously added.

The mercurial liquids of PACINI (§ 400) used to be considered good. HAYEM (op. cit.; see also *Zeit. f. wiss. Mik.*, vi, 3, 1889, p. 335) has a similar formula, viz. sublimate 0·5, salt 1, sulphate of soda, 5, and water 200. This should be mixed with blood in the proportion of about 1:100. Eosin may be added to it. LÖWIT's formula (*Sitzb. k. Akad. Wiss. Wien*, xcv, 3, p. 129; *Zeit. f. wiss. Mik.*, vi, 1, 1889, p. 75) consists of 5 c.c. cold saturated sublimate solution, 5 grms. sulphate of soda, 2 grms. salt, and 300 c.c. water. Mosso finds, however, that both of these are too weak in sublimate.

Of course other well-tried fixing fluids, such as Flemming's solution, or Hermann's, may also be used for blood.

LAVDOWSKY (in a long paper in *Zeit. f. wiss. Mik.*, x, 1, 1893, p. 4) describes some remarkable results obtained by fixing with 2 per cent. iodic acid, and staining with Neu-Victoriagrün, methyl violet 6 B, or gentian violet, a process which is said to reveal the presence of nuclei in elements generally considered to be apyrenematous.

812. Stains for Blood.—Blood prepared as above can be satisfactorily stained with many of the usual reagents.

Eosin stains rose-red all parts of blood-corpuscles that contain hæmoglobin (see WISSOWSKY, *Arch. f. mik. Anat.*, 1876, p. 479); parts that do not contain hæmoglobin, such as the nucleus, remaining unstained. This suggests double-staining with eosin and hæmatoxylin.

WISSOWSKY (l. c.) stains in a solution of equal parts of eosin and alum in 200 parts of alcohol, and then with hæmatoxylin.

MOORE (*The Microscope*, 1882, p. 73; *Journ. Roy. Mic. Soc.*, 1882, p. 714) stains for three minutes in a similar solution without the alum, washes, and stains for two minutes in a 1 per cent. aqueous solution of methyl green. Red corpuscles, red; nuclei and white corpuscles, bluish green.

The liquid of CHENZINSKY has been given (§ 315). It stains nuclei and eosinophilous granules.

MERKEL'S *carmine and indigo-carmine* stain has been discussed above (§ 340).

Fresh (unfixed) blood is perhaps best treated as follows (BIZZOZERO and TORRE, *Archivio per le Scienze mediche*, vol. iv, No. 18, 1880, p. 390):—Dilute a drop of blood with 0·75 per cent. salt solution in which has been dissolved a little *methyl violet*. This liquid in no wise affects the form of the elements, stains intensely the nucleus of the red corpuscles, and, in the white, stains the nucleus intensely, and the protoplasm less intensely. May be used for the study of bone-marrow and spleen.

For the staining of the blood-plates of BIZZOZERO, this observer (*Arch. f. path. Anat. u. Phys.*; *Zeit. f. wiss. Mik.*, 1884, p. 389) employs a 0.02 per cent. solution of methyl violet in salt solution, or a 1 : 3000 solution of gentian violet.

Toison (*Journ. Sci. med. de Lille,* fév., 1885; *Zeit. f. wiss. Mik.*, 1885, p. 398) recommends that blood be mixed with the following fluid:

Distilled water	160 c.c.
Glycerin (neutral, 30° Baumé) .	30 ,,
Pure sulphate of sodium . .	8 grammes.
Pure chloride of sodium . .	1 gramme.
Methyl violet 5 B . . .	0·25 ,,

(The methyl violet is to be dissolved in the glycerin with one half of the water added to it; the two salts are to be dissolved in the other half of the water, and the two solutions are to be mixed and filtered.) White blood-corpuscles stain in this medium in five or ten minutes; the maximum of coloration is attained in from twenty to thirty minutes. White blood-corpuscles, violet; red blood-corpuscles, greenish.

Ferrier's liquid is said to have a sp. gr. similar to that of liquor sanguinis. Fuchsin, 1 grm.; water, 150 c.c.; rectified spirit, 50 c.c.; dissolve, and add glycerin, 200 c.c. (from Squire's *Methods and Formulæ,* p. 39).

Leclerq's fuchsin followed by malachite green, or Congo followed by gentian and eosin, see *Bull. Soc. Belge de Mic.*, xvi, 1890, p. 61: or *Journ. Roy. Mic. Soc.*, 1890, p. 675.

Dekhuysen's methylen blue and acid fuchsin mixture, see *Verhandl. Anat. Gesellsch.*, 1892, p. 90; or *Journ. Roy. Mic. Soc.*, 1893, p. 116.

It goes without saying that the Ehrlich-Biondi mixture, (§ 306), and Ehrlich's triacid and acidophilous mixtures, (§§ 307 and 323) will be found most valuable reagents in many hæmatological researches. Löwit (Ziegler's *Beitr. z. path. Anat.*, &c., x, 1891, p. 214; *Zeit. f. wiss. Mik.*, viii, 3, 1891, p. 371) obtained instructive results by staining sublimate preparations for one to two minutes in a *concentrated* Ehrlich-Biondi solution, and examining in water or glycerin.

For details as to the reactions of the granules of leucocytes and of Löwit's "pyrenogenous" corpuscles, see the original paper; also Ehrlich's "Methodologische Beitr. z. Physiol., &c., der Leucocyten," in *Zeit. f. klin. Med.*, i, 1880, 3, p. 558; cf. *Zeit. f. wiss. Mik.*, i, 1884, p. 382, and later papers of Löwitz in *Anat. Anz.*, vi, 1891, p. 344, and *Arch. f. mik. Anat.*, xxxviii, 1891, p. 524 (*Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 233). Also, Lovell

Gulland (*Journ. of Physiol.*, xix, 1896, p. 385). For some details concerning the carrying out of Ehrlich's cover-glass preparation method, see Reinbach, in *Arch. f. klin. Chirurg.*, xlvi, 1893, p. 486 (*Zeit. f. wiss. Mik.*, xi, 2, 1894, p. 258). For the process employed by Foà (*Festschr. R. Virchow gewidm.*, &c., 1891, i, p. 481; *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 227), see § 352.

The elaborate paper of Müller mentioned in § 810 is too rich in detail to bear abstracting here. A novelty in it is the impregnation of cover-glass preparations with gold chloride by Ranvier's formic acid process.

813. Weigert's **Fibrin Stain** (*Fortschr. d. Med.*, v, 1887, No. 8, p. 228; *Zeit. f. wiss. Mik.*, iv, 4, 1887, p. 512). Sections (alcohol material) are stained in a saturated solution of gentian or methyl violet in anilin water (§ 278). They are brought on to a slide and mopped up with blotting-paper, and solution of LUGOL is poured on to them. After this has been allowed to act for a sufficient time they are differentiated and cleared in anilin oil *without previous dehydration with alcohol.* They are simply mopped up with blotting-paper, and a drop of anilin is poured on to them. The anilin soon becomes dark, and is then changed for fresh once or twice. The sections are by this means differentiated and cleared at the same time. When this has been done, the anilin is *thoroughly* removed by means of xylol, and a drop of balsam and a cover are added. This stain may be applied to celloidin sections without previous removal of the celloidin. Fibrin is sharply stained in blue, bacteria and fungi are also stained of a very dark blue. See also the modifications of this method by Kromayer (§ 665) and Benecke (§ 792).

814. Demonstration of Blood-plates of Bizzozero (Kemp, *Studies fr. the Biol. Lab. Johns Hopkins Univ.*, May, 1886, iii, No. 6; *Nature*, 1886, p. 132).—The mere demonstration of the blood-plates of Bizzozero is easy enough. A somewhat large drop of blood is placed on a slide, and quickly washed with a small stream of normal salt solution. The blood-plates are not washed away, because they have the property of adhering to glass; and on bringing the slide under the microscope they will be seen in large numbers. If it be desired to make permanent preparations of them, they should first be fixed. This is done by putting a drop of osmic acid solution on the finger before pricking it.

For BIZZOZERO's recent methods for the *numeration* of these elements and for the study of their regeneration, see his paper in *Festschr. R. Virchow gewidm.*, &c., 1, 1891, p. 459; or the report of the methods in *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 229.

For the application of some *digestion methods* to the study of blood-plates, see LILIENFELD, *Arch. f. Anat. u. Physiol.*, Physiol. Abth., 1892, p. 115; or *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 363.

For methods for obtaining *large quantities* of blood-plates, see DRUEBIN, *Die Herstellung wägbarer Mengen von Blutplättchen*, Jurjew, 1893; *Zeit. f. wiss. Mik.*, x, 4, 1893, p. 493.

815. BIONDI's **Section Method for Blood** (*Arch. f. mik. Anat.*, xxxi, 1888, p. 103).—A process of imbedding in agar-agar. See last edition, or *Journ. Roy. Mic. Soc.*, 1888, pp. 313, 659. SCHIEFFERDECKER says that celloidin may be employed (see his *Gewebelehre*, p. 389).

Glands.

816. Mucin.—HOYER, who has made a special study of the staining reactions of mucin in tissues (*Arch. f. mik. Anat.*, xxxvi, 1890, p. 310; see also *Zeit. f. wiss. Mik.*, viii, 1, 1891, p. 67), has the following conclusions:

The mucin of mucus cells and goblet cells, both of Vertebrates and Invertebrates, stains with *basic* tar colours, but not with *acid* tar colours (see above, § 268). More or less specific stains of mucin are obtained, for instance, with hydrochlorate or nitrate of rosanilin, commercial fuchsin, Grübler's "neutral" fuchsin ("*n. Unna*"), magenta, Magdala red, iodine green, methyl green, dahlia, methyl violet, gentian, iodine violet, crystal violet, Victoria blue. A similar reaction is obtained with alum-hæmatoxylin solutions, whilst carmine behaves like the acid coal-tar dyes, and affords no stain.

HOYER obtained his best results by means of thionin (violet of Lauth), which gives a double stain, the tissues blue, the mucin elements ruddy violet. The dye called amethyst, prepared by Geigy and Co., of Bâle, is a good succedaneum, and so are toluidin blue (obtainable from Grübler) or the phenylen blue of Oehler in Offenbach, and the p-phenylen blue of the Höchst manufactory. Like thionin, all these give metachromatic stains.

Results less brilliant than those given by the above-mentioned stains, but nevertheless excellent, are obtained by means of methylen blue or Bismarck brown. Methylen green and safranin also give good reactions, but are somewhat inconstant in their effects. Methylen blue is particularly useful from its power of bringing out the merest traces of mucin.

All of these colours may be used in the same way. Specimens should be fixed for two to eight hours in 5 per cent. sublimate solution, imbedded in paraffin, cut, and the sections stained for five to fifteen minutes in a very dilute aqueous solution of the dye (two drops of saturated solution to 5 c.c. of water).

It is theoretically interesting to observe that hyaline cartilage, the jelly of Wharton, and the Mastzellen of Ehrlich give the same reactions with basic dyes as mucin does, even their metachromatic reactions being identical.

These conclusions had already been in part formulated by SUSSDORF (*Deutsche Zeit. f. Thiermed.*, xiv, pp. 345, 349; see *Zeit. f. wiss. Mik.*, vi, 2, 1889, p. 205).

See also the important series of papers by BIZZOZERO, "*Sulle ghiandole tubulari del tubo gastro-enterico*," &c., in the *Atti R. Accad. di Sci. di Torino*, 1889 to 1892; reports in *Zeit. f. wiss. Mik.*, vii, 1, 1890, p. 61; and ix, 2, 1892, p. 219. As regards the safranin reaction, it is well to note that it is not obtained with all brands of the dye; that of Bindschedler and Busch, in Bâle, gives it, whilst safranin 0 of Grübler does not. For the distinctive reactions of old and young mucin see the original, or the last-quoted report of the *Zeit. f. wiss. Mik.*

The subject has recently been carefully investigated by PAUL MAYER ("Ueber Schleimfärbung," *Mitth. Zool. Stat. Neapel*, xii, 2, 1896, p. 303). As regards the hæmatein reaction, he establishes the following propositions.

1. If the staining solution contain free acid, as in Mayer's acid hæmalum or Ehrlich's hæmatoxylin, then as a general rule the secretion of mucus gland cells does *not* stain in it.

2. If it contain a relatively large proportion of alum, say 5 per cent., as in hæmalum, or more, then also as a general rule the secretion of mucus gland cells does *not* stain in it.

3. If it contain a relatively small proportion of alum, but a large proportion of hæmatein, then it stains many sorts of mucus, and at the same time stains chromatin strongly.

As the result of his investigations Mayer gives the following formulæ for specific stains of the secretion of mucus cells (the distinction between mucin and mucigen is not taken in this paper).

Mucicarmine.—One gramme of carmine is rubbed up in a capsule with 0·5 gramme of aluminium chloride (must be dry, not damp and yellow), and 2 c.c. of distilled water. The capsule is heated over a small flame for two minutes, until the originally light-red mixture has become quite dark. Stir thoroughly. The liquid having become thick, add a little 50 per cent. alcohol, in which the warm mass ought to dissolve easily, and rinse the whole with more alcohol into a bottle. Make up to 100 c.c. with 50 per cent. alcohol, let it stand for at least twenty-four hours, and filter. This gives a stock solution, which is as a rule to be diluted for use tenfold with distilled or tap water. Exceptionally it may be diluted instead five or ten fold with alcohol of 50 per cent. or 70 per cent. The stock solution may be obtained from Grübler and Co. Mucicarmine stains in sections or thin membranes *mucus only.*

Muchæmatein. — Hæmatein 0·2 g., aluminium chloride 0·1 g., glycerin 40 c.c., water 60 c.c. Rub up the hæmatein in a mortar with a few drops of the glycerin, then add the other ingredients. If it be desired to avoid employing a watery liquid, an alcoholic solution may be made in the same way by dissolving the hæmatein and aluminium chloride in 100 c.c. of 70 per cent. alcohol, with or without the addition of two drops of nitric acid. Either of these solutions is a *nearly pure mucus stain* for sections or thin membranes.

Mayer's paper contains such other matter not abstracted here, which will well repay careful study. A fairly detailed abstract may be found in *Zeit. f. wiss. Mik.*, xiii, 1, 1896, p. 38.

For the methods of Unna for obtaining a specific stain of mucin by means of polychromatic methylen blue, see *ibid.*, p. 42, or the original, *Monatsh. f. prakt. Dermatol.*, xx, 1895, p. 365.

817. Goblet Cells.—So far as these contain mucin they give the reactions above described (see Flemming, *Zeit. f. wiss. Mik.*, 1885, p. 519; and Paulsen, *ibid.*, p. 520). But the reactions appear to be different for different animals. Thus Paneth (*Arch. f. mik. Anat.*, xxxi, 1888, p. 113, *et. seq.*)

found that in the small intestine of the mouse the contents of the goblet cells did not stain with Böhmer's hæmatoxylin. And the goblet cells of the small intestine of man did not stain with safranin.

Ranvier, in a paper too long to be abstracted here (*Comptes rend.*, 1887, 3, p. 145; see also *Zeit. f. wiss. Mik.*, v, 2, 1888, p. 233), describes a specific reation of perruthenic acid (RuO_4) on goblet cells. By treating the pharyngeal mucosa of the frog first for ten to twelve hours with vapour of osmium, and then for three minutes with vapours of perruthenic acid, the goblet cells are brought out with remarkable distinctness. The contained mucigen is stained black, but the vacuoles are unstained. Since perruthenic acid is very rapidly reduced by organic matter, Ranvier regards this reaction as a proof that the vacuoles do not contain any organic substance, but probably only water and inorganic salts.

For detailed instructions for the study of goblet cells, see List, in *Arch. f. mik. Anat.*, xxvii, 1886, p. 481.

818. Salivary Glands.—Solger (*Unters. z. Naturlehre d. Menschen*, xv, 5 and 6, pp. 2—15; *Zeit. f. wiss. Mik.*, xii, 3, 1896, p. 374) demonstrates the granules in serous cells and half-moons of the submaxillary gland by means of formaldehyde. The gland is hardened for two days or more in a 10 per cent. solution of formol, and may then either be sectioned and examined in the wet way or imbedded in paraffin, and the sections stained with hæmatoxylin of Delafield or of Ehrlich, the granules taking the stain. See also § 348.

819. Liver.—See hereon the important papers of Ranvier, "*Les membranes muqueuses et le syst. glandulaire*," in the *Journ. de Microgr.*, ix, x, 1885–6; Igacuschi, in *Arch. f. path. Anat.*, xcvii, p. 142, or *Zeit. f. wiss. Mik.*, 1885, p. 243 (gold process for study of fibrous networks); Kupffer, *Sitzb. Ges. f. Morph.*, &c., München, Juli, 1889, or *Zeit. f. wiss. Mik.*, vi, 4, 1889, p. 506 (hæmatoxylin stain for demonstration of ultimate bile-ducts, and application of Golgi's silver bichromate method to the same object and to the study of fibrous networks); Oppel, *Anat. Anz.*, v, 1890, p. 143; vi, 1891, p. 165; and *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 222; viii, 2, 1891, p. 224 (also concerning the application of Golgi's process to the above objects).

820. Other Methods for Glandular Structures.—Amongst numerous important papers that cannot be quoted here, see Ranvier, "*Le mécanisme de la sécrétion,*" in *Journ. de Microgr.*, x, 1886–7, and the valuable papers of Heidenhain in Pflüger's *Archiv.*

The peculiar applicability of the Ehrlich-Biondi stain to this kind of work hardly needs pointing out.

CHAPTER XXXV.

SOME ZOOLOGICAL METHODS.

821. Introduction.—It is the purpose of this chapter to describe such variations of the usual histological processes as are indicated for the study of organisms which offer special difficulties. The methods described are all of them such as give results applicable to histological study, and no account has been taken of such methods as are merely useful for the preparation of organisms for museum specimens or for coarse dissection. But of course in many cases the methods recommended for histological work will be found to give admirable results for the preparation of show specimens, and may be used for that purpose if desired.

A further word here as to the employment of formaldehyde. The introduction of this reagent has singularly facilitated museum work. I consider it to be established that as a *preservative* agent for delicate organisms, it is in many cases greatly superior to alcohol. For as there is not even a partial dehydration, all risk of shrinkage on that head is done away with. But it is only, I think, as a *preservative* agent that it offers signal advantages. As a *fixing* agent, even for mere museum specimens, it will only, I think, be found to give good results in a very few cases. In the vast majority of cases, organisms should first be fixed *secundum artem,* in one of the tried and appropriate fixing agents, and then, after proper washing if necessary, be transferred to solution of formaldehyde for preservation.

A valuable paper giving an account of a number of the processes employed in the Naples Zoological Station for the preservation of marine animals has been published by SALVATORE LO BIANCO in *Mitth. Zool. Stat. Neapel,* ix, 1890, p. 435. References to the work of S. LO BIANCO in the remainder of this chapter are to that paper. An abstract of it is contained in *Amer. Natural.*, xxiv, 1890, p. 856, and *Journ. Roy. Mic. Soc.*, 1891, p. 133, and a very full account in *Zeit. f. wiss. Mik.*, viii, 1, 1891, p. 54.

Tunicata.

822. Fixation of Tunicata.—A method of SALVATORE LO BIANCO for killing simple Ascidians in an extended state has been given above, § 22. In the paper quoted above this plan is recommended for *Ciona, Ascidia,* and *Rhopalea.* But many other forms, such as *Clavellina, Perophora, Phallusia, Molgula, Cynthia,* &c., should first be narcotised by treatment for from three to twelve hours with chloral hydrate (1 : 1000 in sea water), then killed in a mixture containing chromic acid of 1 per cent. 10 parts, acetic acid 100 parts, and finally hardened in 1 per cent. chromic acid. The small proportion of chromic acid in the above mixture is stated to be sufficient to neutralise the swelling action of the acetic acid.

The compound Ascidians with contractile zooids are difficult to manage if one does not go the right way to work. The best process known to me is the following (due to VAN BENEDEN, kindly communicated to me by Dr. C. Maurice). Place the corms in clean sea water, and leave them alone for a few hours, in order that the zooids may become fully extended. Seize the corms with your fingers, and plunge them suddenly into glacial acetic acid. Leave them there for two, four, or six minutes, according to the size of the corms (which, of course, you will have taken care to select of as small a size as possible). Take them out of the acid with your fingers (or in some manner that may dispense with the employment of steel instruments, which would blacken the tissues) and bring them into 50 per cent. alcohol. Wash them thoroughly in that, and then bring them in the usual way through successively stronger alcohols.

I most strongly recommend this process, which gives admirably preserved preparations quite free from any opacity either in the tissues or the tunic. The acid will not hurt the fingers if they be washed immediately.

S. LO BIANCO recommends for this group the chloral hydrate process, followed by fixation with sublimate or chromo-acetic acid.

Small pelagic Tunicates are very easily fixed with osmic acid or acid sublimate solution, with the exception of *Anchinia.* The not very numerous preparations I have made of this exceedingly delicate form have all been unsatisfactory. And some other similar forms may be found difficult. I have had a striking failure with *Salpa virgula,* which I fixed with "Flemming," and got a very poor preparation. The very similar *S. pinnata* is fixed perfectly in this medium.

Molluscoida.

823. Bryozoa.—For some methods of killing and fixing see §§ 11, 18, and 19. S. Lo Bianco employs for *Pedicellina* and *Loxosoma* the chloral hydrate method, fixing with sublimate. For *Flustra, Cellepora, Bugula, Zoobothrium,* he employs the alcohol method of Eisig, § 16. For *Cristatella,* see § 17.

Mollusca.

824. Fixation of Mollusca.—Two groups at least amongst the Mollusca offer considerable difficulties in the way of fixation—Lamellibranchiata and Gastropoda.

If it be attempted to take living and normal Lamellibranchiata from the water they are contained in, in order to throw them into a fixing solution, they invariably withdraw their siphon and foot, shut their valves, and die in a state of contraction. And if it be attempted to open the shell by force after death, the mantle is generally injured, and it is impossible to get the foot and siphon into the extended state. De Castellarnau (*La Estacion Zoolog. de Napoles,* Madrid, 1885) advises that they be killed by the method of Eisig and Andres described for Actiniæ in § 16. Before dying, the animals protrude largely their feet, siphons, branchiæ, and tentacles, and die with their shells open. They may be fixed as soon as insensibility has supervened, by bringing them into picro-sulphuric acid, or some other rapidly killing fixing agent.

The same methods recommended for Lamellibranchiata sometimes give good results with Gastropoda. The asphyxiation method has been described in § 23.

S. Lo Bianco advises that Lamellibranchiata, Prosobranchiata, and, amongst the Heteropoda, Atlantidæ, be narcotised with 70 per cent. alcohol, § 16. Opisthobranchiata ought not to give much trouble, and I recommend sudden killing with liquid of Perényi, or the acetic acid method, § 822. *Aplysia* may first be narcotised by subcutaneous injection of about 1 c.c. of a 5 to 10 per cent. solution of hydrochlorate of cocaïn (Robert, *Bull. Scient. de la France*, &c., 1890, p. 449; *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 216).

For *Ancylus*, André (*Rev. Suisse de Zool.*, 1, 1893, p. 429) recommends boiling water.

For Pteropoda in general, liquid of Perényi. *Creseis* is a difficult form. S. Lo Bianco advises the alcohol method, § 16.

Note the hydroxylamin method of Hofer, § 20.

For preservation it may be noted that for Heteropoda and Pteropoda, formaldehyde (preceded by due fixation in a chromic or sublimate solution) is an admirable medium, so far at least as macroscopic appearances are concerned, and for this purpose superior to alcohol.

825. Terrestrial Gastropods.—The asphyxiation method has been described in § 23.

The quantity of mucus that exists in the integument of Gastropoda is often a serious obstacle in the way of preparation. Marchi (*Arch. f. mik. Anat.*, 1867, p. 204) finds that if a living Limax be thrown into moderately concentrated salt solution it will throw off enormous quantities of mucus, and die in a few hours. The epidermis will be found well preserved. If the animal be thrown into osmic acid or Müller's solution, if I understand the writer justly, no secretion of mucus will occur.

826. Eyes of Gastropoda (Flemming, *Arch. f. mik. Anat.*, 1870, p. 441).—The first difficulty here is to obtain the excision of an exserted eye. It is impossible to sever the exserted peduncle in a living animal without its retracting at least partially before the cut is completed. Never mind that; make a rapid cut at the base, and throw the organ into very dilute chromic acid, or 4 per cent. bichromate; after a short time it will evaginate, and remain as completely erect as if alive. Harden in 1 per cent. osmium, in alcohol, or in bichromate.

Carrière (*Zool. Anz.*, 1886, p. 221) gives the following instructions:—Remove the eye, together with a portion of the tentacle, and fix it by exposing it for some minutes to vapour of osmium. Make sections according to the usual methods and fix them on a slide with Schällibaum's collodion. Stain them with picro-carmine; or first depigment them by very careful treatment with *very dilute* eau de Javelle, and then stain with picro-carmine. Mount in dammar. Successful preparations show the tissues perfectly preserved; but Carrière has only been able to make the depigmentation process succeed with *Helix pomatia;* with Prosobranchiata he failed.

827. Eyes of Cephalopoda and Heteropoda (GRENACHER, *Abh. naturf. Ges. Halle-a.-S.*, Bd. xvi; *Zeit. f. wiss. Mik.*, 1885, p. 244).—Fix in picro-sulphuric acid, or in a saturated solution of corrosive sublimate in picro-sulphuric acid (this mixture is especially useful for *Octopus, Eledone,* and *Sepia,* but does not succeed with the pelagic forms, such as *Loligo, Ommatostrephes,* and *Rossia*). Depigment the specimens with hydrochloric acid (in preference to the nitric acid used by Grenacher in former researches). The mixture § 599 may also be used. The operation of depigmentation may be combined with that of staining; if you stain with borax-carmine and wash out in the last-mentioned mixture the pigment will be found to be removed quicker than the stain is washed out. But this process is delicate, and requires a practised hand. The operation of depigmentation may be carried out on sections, but it is better to use portions of retina of 2 to 5 mm. in thickness. Grenacher mounted his preparations in castor oil, see § 447.

Similar methods are recommended by the same author for the eyes of **Heteropoda** (see *Abh. naturf. Ges. Halle-a.-S.*, 1886; *Zeit. f. wiss. Mik.*, 1886, p. 243).

828. Eyes of Chitonidæ (MOSELEY, *Quart. Journ. Mic. Sci.*, 1885, p. 40).—Moseley worked by decalcifying the shell with nitric acid of 3 to 4 per cent. and making sections.

829. Eyes of Pecten and other Forms, see PATTEN, in *Mitth. Zool. Stat. Neapel*, vi, 4, 1886, p. 733.

830. Shell.—Sections of non-decalcified shell are easily obtained by the usual methods of grinding, or, which is often a better plan, by the methods of v. Koch or Ehrenbaum, §§ 174, 175. For sections of decalcified shell, MOSELEY, who has had great experience of this kind of work, particularly recommends the method of decalcification given above, § 828.

831. Injection of Acephala (FLEMMING, *Arch. f. mik. Anat.*, 1878, p. 252).—To kill the animals freeze them in a salt-and-ice mixture, and throw them for half an hour into lukewarm water. They will be found dead, and in a fit state for injection. Chloroform and ether are useless (but see § 20). The injection-pipe may be tied in the heart; but when this has been accomplished there remains the problem of occluding cut vessels that it is impossible to tie. To this end, after the pipe has been tied, the entire animal is filled and covered up with plaster of Paris. As soon as the plaster has hardened the injection may be proceeded with.

832. Maceration Methods for Epithelium of Mollusca.—For the study of ciliated epithelium the following methods are recommended by ENGELMANN (*Pflüger's Arch.*, xxiii, 1880, p. 505):

Cyclas Cornea (intestine), maceration in osmic acid of 0·2 per cent. (after having warmed the animal for a short time to 45° to 50° C.). Also, concentrated boracic acid solution.

The Intra-cellular Processes of the Cilia.—The entire intracellular fibre apparatus may be *isolated* by teasing fresh epithelium from the intestine of a Lamellibranch (*e. g.* Anodonta) in either bichromate of potash of 4 per cent., or salt solution of 10 per cent. To get good views of the apparatus *in situ* in the body of the cell, macerate for not more than an hour in concentrated solution of boracic or salicylic acid. Very dilute osmic acid (*e.g.* 0·1 per cent.) gives also good results. The "lateral cells" of the gills are best treated with strong boracic acid solution (5 parts cold saturated aqueous solution to 1 part water).

BELA HALLER'S **Mixture**, see § 554.

BROCK'S **Medium**, § 549.

MÖBIUS'S **Media**, § 550; the second of these is much recommended by DROOST (*Morphol. Jahrb.*, xii, 2, 1866, p. 163) for *Cardium* and *Mya*.

See also the media recommended by PATTEN (*Mitth. Zool. Stat. Neapel*, vi, 4, 1886, p. 736). Sulphuric acid, 40 drops to 50 grammes of water, is here recommended as a most valuable macerating and preservative agent. Entire molluscs, without the shell, may be kept in it for months.

Arthropoda.

833. General Methods for Arthropoda.—It may safely be stated that, as general methods for the study of chitinous structures, the methods worked out by Paul Mayer (see §§ 7 and 9, and also 75, 240, and 241) are superior to all others. It is absolutely necessary that all processes of fixation, washing, and staining should be done with fluids possessing great penetrating power. Hence picric acid combinations should in general be used for fixing, and alcoholic fluids for washing and staining. *Concentrated* picro-sulphuric acid is the most generally useful fixative, 70 per cent. alcohol is the most useful strength for washing out, and tincture of cochineal in alcohol of 70 per cent. (§ 241) is a very generally useful staining fluid. Mayer's hæmacalcium (§ 257) may sometimes be preferable, and alcoholic carmine and borax-carmine will occasionally give more satisfactory results.

Alcoholic picro-sulphuric acid may be indicated for fixing in some cases.

Some forms are very satisfactorily fixed with sublimate. Such are the Copepoda and the larvæ of Decapoda. It is sometimes indicated to use the sublimate in *alcoholic* solution. Some Copepoda, however (*Copilia, Sapphirina*), are better preserved by means of weak osmic acid, and so are the Ostracoda. In many cases the osmic acid will produce a sufficient differentiation of the tissues, so that further staining may be dispensed with; *Copilia* and *Phyllosoma* are examples of forms that may be prepared in this simple manner. The pyrogallic process (§ 377) may often prove helpful in the study of such forms.

For Ostracoda, Müller (*Fauna u. Flora d. Golfes von Neapel*, xxi (Ostracoda) 1894, p. 8) recommends fixing in a mixture of 5 parts of ether and 1 of absolute alcohol, followed by 70 per cent. alcohol.

834. Methods for Clearing and Softening Chitin.—The employment of eau de Javelle or eau de Labarraque, as suggested by Looss, for making chitin transparent and permeable to reagents has been described above, § 570.

List (*Zeit. f. wiss. Mik.*, 1886, p. 212) has obtained good results with Coccidæ by treating them (after hardening) fo eighteen to twenty-four hours with eau de Javelle, diluted with four volumes of water. After washing out with water, the objects may be dehydrated with alcohol and imbedded in paraffin, the chitin being sufficiently softened to allow of their being penetrated and good sections being obtained. You may stain before imbedding, with alum-carmine or picro-carmine (five to six days).

The same methods are applicable to the preparation of the ova of Insecta—for instance, *Periplaneta* (see MORGAN, *Am. Mon. Mic. Journ.*, ix, 1888, p. 234).

835. Other Depigmentation Methods.—Besides the depigmentation processes discussed in Chap. XXVI, the following methods are available.

SAZEPIN'S **Method for Antennæ of Chilognatha** (*Mém. Acad. Imp. St. Petersb.*, xxxii, 9, 1884, pp. 11, 12).—Sazepin treated antennæ that have been dehydrated with alcohol by steeping them in chloroform. The reaction is slow, the chitin becomes gradually less opaque, but the pigment does not entirely disappear. In order to remove the last trace of it, it will be sufficient if a drop of fuming nitric acid be now added to the chloroform. The mixture must be occasionally agitated, in order to prevent the acid from floating on the chloroform. The reaction is complete in twenty-four hours.

Employed in this manner, nitric acid has no injurious action on tissues.

836. Eyes of Arthropods.—LANKESTER and BOURNE (*Quart. Journ. Mic. Sci.*, 1883, p. 180) prepared the eyes of *Limulus* as follows:—Alcohol, turpentine, paraffin; sections made and carefully depigmented under the microscope with nitric acid of 5 to 10 per cent., then mounted in balsam, some after staining with borax-carmine, others unstained. Non-depigmented sections also mounted in the same manner.

HICKSON (*ibid.*, 1885, p. 243) prepared the eye of a fly as follows:—Remove the posterior wall of the head, and expose the rest, with the eyes *in situ*, for twenty minutes to vapour of osmium. Wash for a few minutes in 60 per cent. alcohol. Harden in absolute alcohol. Make sections. To depigment them, mount them on a slide with Mayer's albumen, remove the paraffin with turpentine, treat them with absolute alcohol, and invert the slide over a capsule containing 90 per cent. alcohol to which a few drops of strong nitric acid have been added. Nitrous vapours are freely given off, and the pigment dissolves. The reaction may be stopped at any moment by washing with pure alcohol.

For dissociation preparations, put the eye or the optic nerve for twenty-four hours into 5 per cent. solution of chloral hydrate, tease, and mount in glycerin. If the elements of the teased tissues be fixed to the slide by means of Mayer's albumen, they may be washed with alcohol and stained *in situ*, or they may be depigmented before staining.

The methods of Parker for eyes of scorpions have been given, § 593.

For the eye of *Homarus* see *Bull. Mus. Comp. Zool., Cambridge, U.S.A.*, xx, 1890, p. 1 (*Zeit. f. wiss. Mik.*, viii, 1, p. 82).

In a later paper (*Mitth. Zool. Stat. Neapel*, xii, 1895, p. 1: *Zeit. f. wiss. Mik.*, xii, 4, 1896, p. 496) Parker describes the application of the methylen-blue method to the study of the retina and optic ganglia in Decapods, especially in *Astacus*. He injected 0·1 c.c. of a 0·2 per cent. solution into the ventral sinus. After twelve to fifteen hours the animals were killed, the ganglia quickly dissected out and brought into aqueous solution of corrosive sublimate, which fixes the stain (see § 294). After fixation the preparations were either dehydrated by the methylal method, described in § 294, or in a modification of that process without the methylal. A stock of 8 per cent. solution of corrosive sublimate in absolute alcohol is prepared, and from this are prepared weaker grades of sublimate-alcohol, by dilution with saturated aqueous sublimate solution. For instance, to make 30 per cent. sublimate-alcohol you take 30 c.c. of the absolute alcohol solution and 70 c.c. of the aqueous solution, and so on. The preparations are dehydrated by passing them through a succession of grades of sublimate-alcohol thus prepared, and of the strengths of 30 per cent., 50 per cent., 70 per cent., and 95 per cent., remaining in each for a quarter of an hour. They then come into the 8 per cent. solution in absolute alcohol for an hour, then into a mixture of one part of this solution with one of xylol, remaining there also for an hour, and finally into pure xylol, in which they may remain till imbedded.

For the methods of Purcell for the eyes of Phalangida, see *Zeit. f. wiss. Zool.*, lviii, 1894, p. 1; *Zeit. f. wiss. Mik.*, xii, 1, 1895, p. 44. He has the following stain. The objects are brought for twenty minutes into 50 per cent. alcohol warmed to 45° or 50° C., and saturated with picric acid. The pigment dissolves in this solution and stains the nuclei and some other parts of the rhabdoms, so that no further stain is required.

See also the methods of Viallanes (*Ann. d. Sci. Nat.*, xiii, 1892, p. 354; *Journ. Roy. Mic. Soc.*, 1893, p. 260).

837. Nerve and Muscle of Arctiscoida (Doyère, *Arch. f. mik. Anat.*, 1865, p. 105).—A score or so of *Milnesium tardigradum* are collected (it is well to have a large number, as the process by no means succeeds with all individuals) and put into a test-tube with water that has been deprived of its air by boiling. A drop of oil is run on to the surface of the water, so as thoroughly to exclude the air. After twenty-four to forty-eight hours the animals will be found, not dead, but fixed and extended in a cataleptic state; the circulation of the perivisceral fluid has ceased, the pigment of the cuticle has disappeared or collected into patches that are no hindrance to observation, the entire animal has gained in transparency, and the nervous and muscular systems stand boldly out. The animals are examined in boiled water, unless it be wished to study the phenomena of resuscitation, in which case spring water should be used.

838. Phalangida (Rössler; see previous editions, or *Zeit. f. wiss. Zool.*, xxxvi, 1882, p. 672).

839. Macrotoma plumbea (Sommer; see previous editions, or *Inaug. Diss.*, 1884, p. 4; *Zeit. f. wiss. Mik.*, 1885, p. 234).

840. Bethe's **Stain for Chitin** (*Zool. Jahrb.*, Abth. f. Anat., viii, 1895, p. 544; *Zeit. f. wiss. Mik.*, xii, 4, 1896, p. 498). Desirous of staining the chitinous hairs and plates of the otocyst of *Mysis*, Bethe found advantage in employing a process, borrowed from industrial dyeing, in which anilin black is produced on the tissue itself. Anilin black is a product of the oxidation of anilin hydrochloride. Bethe proceeds as follows:—Series of sections mounted on a slide are put for three or four minutes into a freshly prepared 10 per cent. solution of anilin hydrochloride, to which has been added one drop of hydrochloric acid for every 10 c.c. They are then rinsed in water, and the slide is put with the sections downwards into 10 per cent. solution of bichromate of

REESE LIBRARY OF THE

potash. The stain quickly begins to show itself, but is at first in general not sufficiently intense. The process is then repeated until the desired intensity of stain is obtained, care being taken to rinse the sections well with water after each of the operations, in order to avoid the formation of precipitates. The stain is at first green, but becomes blue in tap water or alcohol containing ammonia.

The same paper contains a hint concerning the preparation of telsons for section cutting. They are put for eight to fourteen days into 40 per cent. alcohol, to which nitric acid is gradually added, so that by the end of that time they have been brought into alcohol containing 20 per cent. of the acid. This softens the chitin, and somewhat breaks down the structure of the otolith, so that good sections through it are occasionally obtained.

Vermes.

841. Cestodes.—This group must of course be chiefly studied by the usual section methods. As pointed out by Vogt and Yung (*Traité d'Anat. comp. prat.*, p. 204), the observation of the living animal may be of service, especially in the study of the excretory system. And, as shown by Pintner, tæniæ may be preserved alive for several days in common water to which a little white of egg has been added.

Lönnberg (*Centralb. f. Bakteriol. u. Parasitenk.*, xi, 1892, p. 89; *Journ. Roy. Mic. Soc.*, 1892, p. 281) has kept *Triænophorus nodulosus*, a parasite of the pike, alive for a month in a slightly acid pepsin-peptone solution containing from 3 to 4 per cent. of nutritive matter, and less than 1 per cent. of NaCl.

Zernecke (*Zool. Jahrb.*, Abth. f. Anat., ix, 1895, p. 92; *Zeit. f. wiss. Mik.*, xii, 4, 1896, p. 494) has employed with success the bichromate of silver impregnation of Golgi. He kills *Ligula* in the osmio-bichromic mixture (4 : 1), impregnates as usual, makes sections in liver, and treats them by the hydroquinon process of Kallius. Besides the peripheral and central nervous system, muscle-fibres, parenchyma cells, and the excretory vascular system are impregnated.

He has also obtained good results by the methylen-blue method.

842. Trematodes (Fischer, *Zeit. f. wiss. Zool.*, 1884, p. 1).—*Opisthotrema cochleare* may be mounted entire in balsam. For sectioning, Fischer recommends imbedding in a mass made by dissolving 15 parts of soap in 17·5 parts of 96 per cent. alcohol. This mass melts at about 60° C., penetrates very rapidly, and solidifies very quickly. The sections should be studied in glycerin.

Wright and Macallum (*Journ. of Morph.*, i, 1887, p. 1) find that *Sphyranura* is for most purposes best fixed in liquid of Flemming, and stained with alum-cochineal.

Cercariæ.—Schwarze (*Zeit. f. wiss. Zool.*, xliii, 1886, p. 45) found that the only fixing agent that would preserve the histological detail of these forms was cold saturated sublimate solution warmed to 35°—40° C.

843. Turbellaria.—Methylen blue will in some cases be found useful for the study of living specimens.

Fixing is difficult, and generally unsatisfactory.

For *Rhabdocœla*, Braun (*Zeit. f. wiss. Mik.*, iii, 1886, p. 398) proceeds as follows:—For preparing entire animals, the specimens are got on to a slide, lightly flattened out with a cover, and killed by running under the cover a mixture of three parts of liquid of Lang with one of 1 per cent. osmic acid solution. Other fixing media than that described were not satisfactory. (Böhmig, however, commenting on this, says that for some of the tissues, such as muscle and body parenchyma, nitric acid and picro-sulphuric acid are very useful.) Sections may be made by the usual paraffin method.

Delage (*Arch. de Zool. exp. et gén.*, iv, 2, 1886; *Zeit. f. wiss. Mik.*, iii, 2, 1886, p. 239; *Journ. Roy. Mic. Soc.*, 1886, p. 1073) strongly recommends fixation (of Rhabdocœla Acœla) by the osmium-carmine mixture, § 237. Concentrated solution of sulphate of iron is also an excellent fixing medium. The animals (*Convoluta*) die in it fully extended. Liquid of Lang was not successful.

For staining, he recommends either the osmium-carmine stain or impregnation with gold (⅓ formic acid, two minutes; 1 per cent. gold chloride, ten minutes; 2 per cent. formic acid, two or three days in the dark. It is well to allow an excessive reduction to take place, and then lighten the stain by means of 1 per cent. solution of cyanide of potassium).

Böhmig, commenting on the above, says that he has obtained very instructive images with Plagiostomidæ fixed with sublimate and stained with osmium-carmine.

Von Graff (*Die Organisation d. Turbellaria Acœla*, Leipzig, 1891; see *Zeit. f. wiss. Mik.*, ix, 1, 1892, p. 76) has the following remarks: Chromo-aceto-osmic acid, followed by hæmatoxylin, is good for the skin; but even this method will not afford a satisfactory preservation of the Rhabdites, which in Acœla and Alloiocœla seem to be destroyed by swelling, whilst in terrestrial and fresh-water Planaria, Polyclada, and most Rhabdocœla they are better preserved. The same method is also good for the parenchyma of *Amphichœrus cinereus*, *Convoluta paradoxa*, and *C. sordida*. Sublimate is not good for these forms, but it is good for *Convoluta Roscoffensis*. For some forms it is important to avoid picro-carmine, which destroys the central parenchyma. The nervous system may be investigated by the methods of DELAGE.

For Dendrocœla sublimate solutions, sometimes hot, appear indicated for fixing. CHICHKOFF (*Arch. de Biol.*, xii, 1892, p. 438; *Journ. Roy. Mic. Soc.*, 1893, p. 262) recommends the following for fresh-water Dendrocœla:—2 per cent. sublimate solution, 6 parts; 15 per cent. acetic acid, 4 parts; pure nitric acid, 2 parts; 14 per cent. chloride of sodium, 8 parts; and 2 per cent. alum, 1 part. The animals are said to die in it without contraction. Note also the mixtures of LANG, § 58. Mayer's tincture of cochineal, § 241, may be found useful for the study of glands, for which purpose the Ehrlich-Biondi stain may also be employed.

844. Nemertina.—After considerable experience of this difficult group I have to say that I know of no method of fixation that will certainly give good results. My best results have always been obtained with cold saturated sublimate solution, acidified with acetic acid. I have tried most of the energetically hardening fixing agents, such as the osmic and chromic mixtures, and do not recommend them for this group, for they seem (the chromic mixtures and perchloride of iron in particular) to act as irritants, and provoke such violent muscular contractions that the whole of the tissues are crushed out of shape by them. And, besides, they do not kill as quickly as sublimate.

I have found it a good plan to decapitate the animals (in the larger forms), cut them up quickly into lengths (not too long), and throw these sharply into the sublimate, the muscular contractions being less energetic in segments that are no longer in connection with the cerebral ganglia.

Perhaps a better method than this will be found in the simple process, suggested to me by Prof. DU PLESSIS, of fixing with hot (almost boiling) water. On the few occasions on which I have tried it the animals have died in extension, without vomiting their proboscis; and I think it is certainly worth trial, especially for the larger forms.

I have tried FOETTINGER's chloral hydrate method (§ 18). My specimens died fairly extended, but vomited their proboscides. According to S. LO BIANCO narcotisation with a solution of 0·1 to 0·2 per cent. in sea water is found successful at Naples.

DE CASTELLARNAU (*Estacion Zool. de Napoles*, p. 137) says that Nemerteans can be successfully narcotised by Eisig's alcohol method, described § 16, and I think the process may be a good one for some of the larger forms.

DENDY (see *Journ. Roy. Mic. Soc.*, 1893, p. 116) has succeeded with *Geonemertes* by exposing it for half a minute to the vapour of chloroform.

Intra vitam staining with methylen blue may be found useful in some cases. For the application of the methylen-blue method to the study of the nervous system see § 290.

For staining fixed specimens *in toto* I hold that it is well-nigh necessary to employ alcoholic stains, for even the most delicate species are not satisfactorily penetrated by watery stains in any reasonable lapse of time. Borax-carmine or Mayer's alcoholic carmine may be recommended; not so cochineal or hæmatoxylin stains, on account of the energy with which they are held by the mucin which in general exists in such great abundance in the skin of these animals.

Sections by the paraffin method, after penetration with oil of cedar (chloroform will fail to penetrate sometimes after the lapse of weeks).

845. Nematodes.—The extremely impermeable cuticle of these animals is a great obstacle to preparation. According to Looss (*Zool. Anz.*, 1885, p. 318) this difficulty may be overcome by treating the animals (or their ova, which are in the same case) with eau de Javelle or eau de Labarraque, in the manner described in § 570.

For fixing, most recent authors recommend sublimate solutions; chromic solutions seem to have a tendency to make the worms brittle.

But according to ZUR STRASSEN (*Zeit. f. wiss. Zool.*, liv, p. 655), *Bradynema rigidum*, a parasite of *Aphodius fimetarius*, ought to be fixed for at least twelve hours in mixture of Flemming.

AUGSTEIN (*Arch. f. Naturg.*, Jahrg. lx, 1, 1894, p. 255; *Zeit. f. wiss. Mik.*, xii, 2, 1895, p. 227) found that for *Strongylus filaria* the best fixing agent was MAYER's picro-nitric acid.

Staining is frequently difficult, and sometimes alcoholic carmine, § 240, is the only thing that will give fair results.

BRAUN (see *Journ. Roy. Mic. Soc.*, 1885, p. 897) recommends that small unstained Nematodes be mounted in a mixture of 20 parts gelatin, 100 parts glycerin, 120 parts water, and 2 parts carbolic acid, which is melted at the moment of using. Canada balsam, curiously enough, is said to sometimes make Nematodes opaque.

846. Demonstration of Living Trichinæ (Barnes, *Amer. Mon. Mic. Journ.*, xiv, 1893, p. 104; *Journ. Roy. Mic. Soc.*, 1893, p. 406).—A piece of trichinised muscle of the size of a pea should be placed in a bottle in a mixture of 3 gr. of pepsin, 2 dr. of water, and 2 minims of hydrochloric acid. The whole should be kept at body temperature for about three hours with occasional shaking. The flesh and cysts being dissolved, the fluid is poured into a conical glass, and allowed to settle; the trichinæ are drawn off from the bottom with a pipette, got on to a slide with water, and examined on a hot stage.

847. Acanthocephali.—It is very difficult to kill Echinorhynci so as to have the animals duly extended and the tissues well preserved. Neither corrosive sublimate nor strong osmic acid will, as a rule, attain this end, even after preliminary intoxication with tobacco smoke or chloroform, the animal thus treated dying contracted.

HAMANN, however (*Jen. Zeit. f. Naturw.*, xxv, 1890, p. 113; *Zeit. f. wiss. Mik.*, viii, 2, 1891, p. 209), has succeeded with sublimate, and also with alcohol containing a little platinum chloride.

SAEFFTIGEN (*Morphol. Jahrb.*, x, 1884, 120; *Journ. Roy. Mic. Soc.* [N.S.], v, 1885, p. 147) obtained the best results by killing gradually with 0·1 per cent. osmic acid; the animals placed in this contract during the first hours, but stretch out again and die fully extended.

Another method of killing is treatment with 0·1 per cent.

chromic acid; Echinorhynci live for days in it, but eventually die fully extended.

KEISER (*Biblioth. Zool.*, H. vii, 1 Hälfte, 1891; *Zeit. f. wiss. Mik.*, viii, 3, 1891, p. 363), found that a saturated aqueous solution of cyanide of mercury, warmed to 45° to 50° C., and allowed to act for from fifteen to sixty minutes, and then washed out with 70 per cent. alcohol, was the best of all fixing media for *Acanthocephali.*

He also found the following mixture excellent:

Picric acid . . .	1 gramme.
Conc. sulphuric acid . .	10 grammes.
Chromic acid . . .	1 gramme.
Water	1000 grammes.

To be warmed to 55° C., allowed to act for fifteen to twenty minutes, washed out for five to ten minutes with hot water, and afterwards for some days in 60 per cent. alcohol.

848. Gephyrea.—VOGT and YUNG (*Anat. comp. prat.*, p. 373) direct that *Siphunculus nudus* be kept for some days in perfectly clean basins of sea water, in order that the intestine of the animals may be got free from sand, which would be an obstacle to section cutting, and then anæsthetised with chloroform, under which treatment they die extended, and may be fixed as desired.

WARD (*Bull. Mus. Comp. Zool., Cambridge, Harvard Coll.*, xxi, 3, p. 144) found the best plan was to put the animals into a shallow dish with sea water and pour 5 per cent. alcohol in a thin film on to the surface of the water. After four to eight hours, if the animals make no contractions on being stimulated, they may be removed to 50 per cent. alcohol.

S. LO BIANCO says killing with 0·5 per cent. chromic acid or with 0·1 per cent. chloral hydrate in sea water may be tried, but either method is uncertain. *Phascolosoma* and *Phoronis* should be treated by the alcohol method.

APEL (*Zeit. f. wiss. Zool.*, xlii, 1885, p. 461) says that *Priapulus* and *Halicryptus* can only be satisfactorily killed by heat. The animals may either be put into a vessel with sea water and be heated on a water-bath to 40° C.; or they may be thrown as rapidly as possibly into boiling water, which paralyses them so that they can be quickly cut open and thrown into one third per cent. chromic acid, or picro-sulphuric acid.

849. Rotatoria.—By far the most important method for the study of this group consists in the observation of the living animals. Great difficulty exists in the way of getting them to keep sufficiently quiet. VOGT and YUNG (*Anat. com. prat.*, p. 420) say that a drop of solution of any of the soluble salts of strychnin run under the cover sometimes renders service. WEBER (*Arch. de Biol.*, viii, 4, 1888, p. 713) finds that strychnin, prussic acid, and curare act too strongly; of all the reagents he tried, 2 per cent. solution of hydrochlorate of cocaïn gave the best results. Warm water gave him good results for large species, such as those of *Hydatina* and *Brachionus.*

HARDY (*Journ. Roy. Mic. Soc.*, 1889, p. 475) recommends thick syrup added drop by drop to the water. HUDSON (*ibid.*, p. 476) mentions weak solution of salicylic acid.

HOFER'S hydroxylamin method has been given, § 20, and TULLBERG'S chloride of magnesium method, § 21; the processes of EISMOND and JENSEN, § 872, may be tried. Methylen blue, § 288, may be found useful.

Permanent preservation of Rotifers has, until lately, been considered by those who have tried it to be well-nigh impossible. Now, however, thanks to the patient experimentation of ROUSSELET, this difficult problem may be considered to be fairly conquered. Rousselet now proceeds as follows (*Journ. Quekett Mic. Club*, v, March, 1895, p. 1): The animals are got together in a watch-glass. They are narcotised by adding to the water at intervals a few drops of the following mixture—

Hydrochlorate of cocaïn, 2 per cent. solution .	3 parts.
Methylated spirit	1 part.
Water	6 parts.

They are watched under a dissecting microscope, and at the moment when the cilia have ceased to beat, or are seen to be on the point of ceasing to beat, they are fixed by adding a drop of liquid of Flemming or of $\frac{1}{4}$ per cent. osmic acid. The fixing agent is allowed to act for half a minute or less, after which the animals are taken out with a pipette, and thoroughly washed by passing them through two or three watch-glasses of distilled water. They are then definitely mounted in 2½ per cent. solution of formaldehyde (formol 2½ parts, distilled water 37½ parts).

For some details concerning variations of this method adapted to the preservation of the different forms, see the paper quoted. Mr. Rousselet has been good enough to show me a large series of preparations made by this method, and I can testify that they are most beautiful.

Annelida.

850. Cleansing Intestine of ***Lumbricus*** (Kükenthal, *Journ. Roy. Mic. Soc.*, 1888, p. 1044).—Put the animals into a tall glass vessel which has been filled up with bits of moistened blotting-paper. They gradually evacuate the earthy particles from the gut, and fill it instead with paper.

Vogt and Yung (*Traité d'Anat. Comp. Prat.*, v) recommend coffee-grounds instead of paper; paper becomes rather hard when imbedded, whereas coffee-grounds cut fairly well.

851. Chætopoda.—*Lumbricus* may be anæsthetised by putting the animals into water with a few drops of chloroform. Perrier has pointed out that it is better not to let the chloroform act directly in solution on the animals, but to put them into water in a shallow dish, set up a watch-glass with chloroform in the corner of it, and cover the whole. In half an hour the worms will be more or less narcotised, and if allowed to remain will die in a state of extension.

Cerfontaine (*Arch. de Biol.*, x, 1890, p. 327; *Zeit. f. wiss. Mik.*, viii, 2, 1891, p. 210) much recommends curare, administered by interstitial injection of a dose of about 2 c.c. of a 1 : 500 solution. The animal should afterwards be put into water, and after a quarter of an hour will be found dead.

In order to kill *Criodrilus lacuum*, Collin (*Zeit. f. wiss. Zool.*, xlvi, 1888, p. 474) puts the animals into a closed vessel with a little water, and hangs up in it a strip of blotting-paper soaked in chloroform. Kükenthal (*Die mik. Technik*, 1885; *Zeit. f. wiss. Mik.*, 1886, p. 61) puts Annelids into a glass cylinder filled with water to the height of 10 centimetres, and then pours 70 per cent. alcohol to a depth of 1 to 2 centimetres on to the water. The animals will be found sufficiently narcotised for fixation in from four to eight hours. For *Opheliadæ* he also employs 0·1 per cent. of chloral hydrate in sea water.

Many marine Chætopoda may be successfully narcotised (S. Lo Bianco) in sea water containing 5 per cent. of alcohol, or by means of the mixture, § **16**.

The *Polychæta sedentaria* offer the difficulty of a complex and very contractile branchial apparatus. They may sometimes be satisfactorily fixed by bringing them rapidly into corrosive sublimate. Cold, not hot solutions should be taken, as heat frequently shrivels up the branchiæ. The species of *Polychæta errantia* that offer a contractile branchial apparatus, as *Eunice* and *Onuphis*, may be treated in the same way.

S. Lo Bianco advises killing Chætopteridæ, Sternaspidæ, *Spirographis, Protula,* by putting them for half an hour into 1 per cent. chromic acid. I have satisfied myself that good show specimens can be obtained in this way; but I doubt the histological preservation of the parts being so good as with sublimate specimens. Some of the *sedentaria* may be got protruded from their tubes by leaving them for some hours in 0·1 per cent. chloral hydrate in sea water (S. Lo Bianco).

See also the methods §§ 18 to 23.

Note also the liquid of Ehlers, § 43.

Staining.—For the staining of small Annelids entire, I find carmalum gives very good results, I think better than borax-carmine or paracarmine.

852. Blood-vessels of Annelids (Kükenthal, *Zeit. f. wiss. Mik.*, 1886, p. 61).—The animals should be laid open and put for two or three hours into *aqua regia* (4 parts of nitric acid to 2 of hydrochloric acid). The ramifications of the vessels will then be found to be stained black, the rest of the preparation yellow.

853. Nerves of Annelids.—The methylen-blue method and the bichromate of silver method of Golgi (the *rapid* method). For the latter see v. Lenhossék (*Arch. f. mik. Anat.*, xxxix, p. 102; *Zeit. f. wiss. Mik.*, ix, 3, 1893, p. 432).

854. Hirudinea.—For the methods of killing see those given for Lumbricus in § 851, also §§ 18 to 23.

Whitman (*Meth. in mic. Anat.*, p. 27) recommends that they be killed with sublimate. This reagent is said to kill leeches with such rapidity that they die in general without having time to change the attitude in which they were found at the moment when the liquid came into contact with them.

I have obtained better results myself by narcotising with carbonic acid (§ 24), and fixing with liquid of Flemming. Small specimens of *Nephelis* are narcotised in a few minutes; large ones will require several hours. I have also found that lemon juice kills them in a state of very fair extension. Carmalum I find excellent for staining entire animals; curiously enough, I have found it to have a better penetration than borax-carmine or paracarmine. Ehrlich-Biondi mixture sometimes gives fine results with sections.

GRAF (*Jen. Zeit.*, 1893, p. 165) states that he has obtained good results by narcotising with decoction of tobacco.

Injection.—WHITMAN (*Amer. Natural.*, 1886, p. 318) states that very perfect natural injections may often be obtained from leeches that have been hardened in weak chromic acid or other chromic liquid. He considers that these injections are the best for the purpose of the study of the circulatory system by means of sections.

Of course Hirudinea (or any other Annelids) on which it is desired to make artificial injections must be killed by some procedure that leaves the tissues in a state that will allow the injection to run freely.

JACQUET (*Mitth. Zool. Stat. Neapel*, 1885, p. 298) advises that leeches be put into water with a very small quantity of chloroform; they soon fall to the bottom of the vessel and remain motionless. They should be allowed to remain a day or two in the water before injecting them.

Echinodermata.

855. Holothurioidea.—These animals are difficult to fix on account of their contracting with such violence under the influence of irritating reagents as to expel their viscera through the oral or cloacal aperture.

S. LO BIANCO puts Holothurids into pure sea water until they have expanded their tentacles, then seizes them with forceps behind the tentacles, so as to mechanically render impossible their withdrawal, and immerses the anterior part of the body in acetic acid, whilst at the same time an assistant injects 90 per cent. alcohol through the anus.

Vogt and Yung (*Anat. Comp. Prat.*, p. 641) say that *Cucumaria Planci* (*C. doliolum*, Marenzeller) is free from the vice of expelling its intestines under irritation; but they recommend that it be killed with fresh water, or by slow intoxication with alcohol, chromic acid, or sublimate added to the sea water in which it is contained.

Synapta may be allowed to die in a mixture of equal parts of sea water and ether or chloroform (S. Lo Bianco).

Holothurids, Dr. Weber informs me, are admirably preserved in formaldehyde; a weak solution is sufficient.

856. Asteroidea.—Hamann (*Beitr. z. Hist. d. Echinodermen*, ii, 1885, p. 2) finds it best to *inject* the living animal with a fixing liquid. The cannula should be introduced under the integument at the extremity of a ray, and the liquid injected into the body-cavity. The ambulacral feet and the branchiæ are soon distended by the fluid, and as soon as it seems to have penetrated sufficiently the animal is thrown into a quantity of the same reagent.

The study of the *eyes* presents points of special difficulty. In order to study them in sections, with the pigment preserved *in situ*, the eye should be removed by dissection, should be hardened in a mixture of equal parts of 1 per cent. osmic acid and 1 per cent. acetic acid, and imbedded in a glycerin gum mass, or some other mass that does not necessitate treatment with alcohol (which dissolves out the pigment, leaving the pigmented cells perfectly hyaline). For maceration use one-third alcohol, the aceto-osmic mixture failing to preserve the rods of the pigmented cells.

Formaldehyde is *not* to be recommended for the preservation of Asteroidea (Weber).

857. Ophiuridea.—Should be killed in fresh water if it be desired to avoid rupture of the rays (De Castellarnau, *La Est. Zool. de Napoles*, p. 135).

858. Echinoidea.—I advise that they be killed by *injection* of some fixing liquid. For preservation, formaldehyde has proved *admirable* in all respects, and greatly superior to alcohol (Weber).

859. Larvæ of Echinodermata.—I am greatly obliged to my able friend Dr. Barrois for kindly writing down for me (for the *Traité des Méth. techn.*, from which they are translated) the following instructions, which are the outcome of a prolonged and minute study of the metamorphoses of the Echinodermata.

Pluteus.—In order to a fruitful study of the metamorphoses of the Echinoidea and Ophiuridea it is necessary to obtain preparations that offer the advantages presented by the study of the living larvæ; and especially such as give distinct images of the different organs, and show the calcareous skeleton preserved intact (a point of considerable importance, since this skeleton frequently affords landmarks of the greatest value). These preparations should further possess the following points:—They should give clear views of the region of formation of the young Echinoderm (which is generally opaque in the living larva). And they should possess sufficient stiffness to allow of the larva being turned about in any desired way, and placed in any position under the microscope.

It is not very easy to obtain preparations fulfilling these conditions, on account of the difficulty of obtaining a selective stain whilst preserving the integrity of the calcareous skeleton. The following method is recommended:—*Pluteus* larvæ are fixed in a cold saturated solution of corrosive sublimate, in which they remain not more than two or three minutes. They are then washed with water, and brought into dilute Mayer's cochineal (§ 241). This should be so dilute as to possess a barely perceptible tinge of colour. The objects should remain in the stain for from twelve to twenty-four hours, being carefully watched the while, and removed from the stain at the right moment and mounted in balsam, or, which is frequently better, in oil of cloves or cedar-wood. This method is perfectly satisfactory for the study of the chief phases of metamorphosis.

Auricularia and Bipinnaria.—The method described above is equally applicable to these forms, and seems to be altogether the best method for the study of the metamorphosis of Bipinnaria. The earlier stages of the metamorphosis of Auricularia are better studied by fixing with osmic acid, staining with Beale's carmine, and mounting in glycerin.

Larvæ of Comatula.—The best method for the study of the embryonal development of *Comatula* consists in fixing with liquid of Lang, and staining with dilute borax-carmine. It is important (for preparations that are not destined to be sectioned) to use only *dilute* borax-carmine, as the strong solution produces an over-stain that cannot easily be reduced.

Narcotisation by chloral hydrate before fixing is useful, especially for the study of *Pentacrinus* larvæ and of the young *Synaptæ* formed from Auricularia. Without this precaution you generally get preparations of larvæ either shut up (*Pentacrinus*), or entirely deformed by contraction (young *Synaptæ*).

See also MacBride on the development of *Amphiura squamata*, *Quart. Journ. Mic. Sci.*, xxxiv, 1892, p. 131; *Journ. Roy. Mic. Soc.*, 1893, p. 117 (osmic acid followed by liquid of Müller and alcohol; decalcification with nitric acid in alcohol; staining with Mayer's paracarmine or hæmalum); and Seeliger on the development of *Antedon*, *Zool. Jahrb.*, Abth. f. Anat., vi, 1892, p. 161; *Zeit. f. wiss. Mik.*, x, 2, 1893, p. 229.

Cœlenterata.

860. Actinida.—*Narcotisation.*—For suitable narcotisation methods see §§ 13 to 23.

Fixation.—In *Le Attinie, Fauna u. Flora d. Golfes v. Neapel*, Andres gives the following hints:—Hot corrosive sublimate often gives good results. In the case of the larger forms the solution should be injected into the gastric cavity, and a further quantity of the liquid be poured over the animals.

Freezing sometimes gives good results. A vessel containing Actiniæ is put into a recipient containing an ice-and-salt freezing mixture and surrounded by cotton wool. After freezing, the block of ice containing the animals is thawed in alcohol or some other fixing liquid.

Maceration.—For the Hertwigs' well-known method (*Jen. Zeit.*, 1879, p. 457) see § 553. The tissues should be left to macerate in the acetic acid for at least a day, and may then be teased in glycerin.

List (*Zeit. f. wiss. Mik.*, iv, 2, 1887, p. 211) recommends dilute liquid of Flemming. Tentacles of *Anthea cereus* and *Sagartia parasitica* treated for ten minutes with a mixture of 100 c.c. of sea water with 30 c.c. of Flemming's liquid (the strong solution, § 47), then washed out for two or three hours in 0·2 per cent. acetic acid, and teased in dilute glycerin, give fine dissociations of the connective, sensory, and urticant cells of the ectoderm, and after removal of the epidermis allow of the demonstration of ganglion-cells and the supporting lamella. Picro-carmine may be used for staining.

861. Zoantharia with Calcareous Skeletons are difficult to deal with on account of the great contractility of the polyps. Sublimate solution, which ought very often to be taken boiling, sometimes gives good results. De Castellarnau (*La Est. Zool. de Napoles*, p. 132) says that this process succeeds well with *Dendrophyllia, Antipathes, Astroides, Cladocora* and *Caryophyllia*.

Sections.—For preparing sections, besides the usual methods for sectioning decalcified specimens, we have the valuable methods of von Koch and Ehrenbaum, §§ 174 and 175, which, being applicable to undecalcified specimens and furnishing preparations showing at one and the same time soft parts and hard parts *in situ*, render most inestimable services.

862. The Alcyonaria have also extremely contractile polyps. In a former edition I suggested for their fixation either hot sublimate solution or glacial acetic acid (§ 68). S. Lo Bianco has since recommended essentially similar processes. Garbini (*Manuale*, p. 151) says that the polyps may be fixed in the state of extension by drenching them with ether, and then bringing them into strong alcohol.

Wilson (*Mitth. Zool. Stat. Neapel*, 1884, p. 3) kills Alcyonaria with a mixture of 1 part of strong acetic acid and 2 parts of concentrated solution of corrosive sublimate, the animals being removed as soon as dead and hardened for two or three hours in concentrated sublimate solution.

Schultze (*Biol. Centralb.*, 1887, p. 760) says that for Pennatulidæ with large polyps the gradual addition of fresh water is a good plan.

863. Zoantharia and Alcyonaria.—Braun (*Zool. Anz.*, 1886, p. 458) recommends that for both Zoantharia and Alcyonaria a little osmic acid be added to the sublimate employed for fixation. For *Alcyonium palmatum, Sympodium coralloides, Gorgonia verrucosa, Caryophyllia cyathus*, and *Palythoa* *axinellæ* he proceeds as follows :—The animals are left for a day or two in a glass vessel, so that the polyps may become thoroughly extended. They are then suddenly drenched with a mixture of 20 to 25 c.c. of concentrated solution of sublimate in sea water with four to five drops of 1 per cent. osmic acid. This is allowed to act for five minutes.

(This method also gives good results with *Hydra* and some Bryozoa and Rotifers.)

864. Hydroidea, Polypoid Forms.—For suitable *narcotisation* methods see those quoted in last section.

Fixation.—In general the polyps may be very well killed in saturated sublimate solution, in which they should be plunged for an instant merely, and be brought into alcohol. The solution should be employed cold in general for Gymnoblastea, hot for most Calyptoblastea.

Ether attentively administered gives good results with Campanularidæ. *Hydra* is very easily killed by treatment with a drop of osmic acid on a slide. BRECKENFELD (*Amer. Mon. Mic. Journ.*, 1884, p. 49) obtains good results by heating the animals in a drop of water on a slide for from three to five seconds over a petroleum lamp. The methods for sections are the usual ones.

The *methylen-blue* method of *intra vitam* staining is applicable to this group, see ZOJA, l. c., § 288.

865. Medusæ : Fixation.—There is some difficulty in properly fixing the forms with contractile tentacles, which easily roll up on contact with reagents. The best results I have had with these forms have been obtained by means of VAN BENEDEN'S acetic acid method, § 68, followed by alcohol. The secret of success with the long-tentacled forms lies in a trick of manipulation. Put sufficient acetic acid into a deepish dish, hold it in your left hand (or, better, in both hands if you have an assistant), and keep it moving in a circle so as to communicate a vortex motion to the liquid. Take up a medusa in a spoon with as little sea water as possible, and throw it into the moving liquid, and keep the liquid steadily swirling round so as to cause the tentacles to trail out at full length behind the animal until it is thoroughly fixed, then pass carefully into alcohol. Do not, unless you are very expert, try to fix more than one medusa at a time; it is also better to keep the specimens separate, even in the alcohol, as, if several are together, it generally happens that their tentacles become entangled. The method is due to S. Lo Bianco. *Oceania conica* and *Tiara* may usefully, according to S. Lo Bianco, be narcotised with 3 per cent. alcohol in sea water before fixation. Liquid of Kleinenberg, which I have seen much used for the fixation of these and similar forms, is, in my opinion, histologically a very objectionable reagent for the purpose.

Trachymedusæ and Acalephæ may be fixed in the usual way in chromic or osmic mixtures. Osmic acid may conveniently in some cases be added to the sea water containing the animals, which should be removed to fresh water as soon as they begin to turn brown. *Cassiopeia borbonica*, according to de Castellarnau, ought to be treated with osmic acid as described, and then put for two or three days into 5 per cent. solution of bichromate of potash. I have tried this process, with good results.

866. Medusæ: Sections.—I am not acquainted with any perfectly satisfactory method of sectioning these extremely watery organisms. Paraffin and collodion will afford good sections of some organs, but are certainly not satisfactory as all-round methods for this group. Some modification of the method employed by the Hertwigs (*Nervensystem der Medusen*, 1878, p. 5) might be successful. They imbedded in liver with the aid of glycerin gum, and hardened the objects and the mass in alcohol. I should think better results would be obtained by one of the freezing methods given in §§ 177 to 184.

867. Medusæ: Maceration.—The methods of the Hertwigs, § 553, have deservedly become classical for the study of the tissues of this group.

Amongst other advantages of this process it should be noted that the reduction of osmic acid by albuminates is greatly hastened by the presence of acetic acid, which in the case of animals so transparent and poor in cells as Medusæ is an advantage for the study of the nervous system; for ganglion-cells and nerve-fibrils reduce osmic acid quicker than common epithelium-cells. They become greenish brown, and are easily distinguished from surrounding tissues. Doubtless in many cases the pyrogallic acid reaction, § 377, would give better results.

The isolation of the elements of the macerated tissues is best done by gently tapping the cover-glass (which may be supported on wax feet). This gives far better results than teasing with needles. A camel-hair pencil also sometimes renders good service.

868. Siphonophora.—This group contains some of the most difficult forms to preserve that are to be found in the whole range of the animal kingdom. You have not only to deal with the very great contractility of the zooids, but with the tendency to general disarticulation of the swimming-bells and prehensile polyps.

The cupric sulphate method of BEDOT (*Arch. d. Sci. phys. et nat.*, Juin, 1889, t. xxi, p. 556) recommended for the preparation of Siphonophora and other delicate pelagic animals, is as follows:—Bedot directs that a large quantity of 15 to 20 per cent. solution of the salt be suddenly added to the sea water containing the animals. As soon as they are fixed (which happens in a few minutes) a few drops of nitric acid are to be added and mixed in (this is in order to prevent the formation of precipitates), and the whole is left for four to five hours. The specimens are then to be hardened *before* bringing them into alcohol. Bedot recommends that this be done with solution of Flemming. The strong solution is the one that should be taken, and it should be *added* to the solution of sulphate containing the Siphonophore, about two volumes of it being taken for one of the sulphate solution. The whole should be left for at least twenty-four hours. After hardening in the mixture a few drops of 25 per cent. alcohol should be added to the fluid with a pipette, being dropped in as far as possible from the colony, which should be disturbed as little as possible; and further alcohol, of gradually increasing strength, should be added so gradually that the strength of 70 per cent. be not attained under fifteen days at least. Ninety per cent. alcohol should be used for definite preservation.

I have tested this method. I do not find that the histological preservation is superior to that obtained by means of the usual processes; but the method is certainly a valuable one in so far as *it enables one to preserve specimens with all their swimming-bells and polyps in situ*, a result which is not obtained by means of the usual methods.

FRIEDLAENDER (*Biol. Centralbl.*, x, 1890, p. 483; *Journ. Roy. Mic. Soc.*, 1890, p. 804) preserves this class of objects by inundating them with a mixture of 125 parts cupric sulphate, 125 parts zinc sulphate, and 1000 parts water.

S. LO BIANCO employs for the majority of Siphonophora a mixture of 10 c.c. of saturated solution of corrosive sublimate with 100 c.c. of 10 per cent. solution of copper sulphate. This is used as in Bedot's process. *Diphyes, Rhizophysa,* and *Physalia,* however, are killed with sublimate solutions; *Velella* with chromo-picric acid, or a mixture of 100 c.c. of sublimate solution with 50 c.c. of 1 per cent. chromic acid; *Porpita* by poisoning with liquid of Kleinenberg.

KOROTNEFF's method of paralysing with chloroform has been given in § 15. I would only add that I have seen *Physophora* very successfully killed by the careful administration of ether.

Preservation, after fixation and washing, is greatly simplified by the use of formaldehyde instead of alcohol. Dr. WEBER has prepared some beautiful specimens at Villefranche by this method.

869. Ctenophora : Fixation.—The small forms are very easily prepared by means of osmic acid. For the large forms see the paper of S. LO BIANCO, quoted § 821.

SAMASSA has succeeded in making sections of Ctenophora by means of the double-imbedding method, § 169 (see *Arch. f. mik. Anat.*, xl, 1892, p. 157; *Zeit. f. wiss. Mik.*, 1893, p. 340).

Porifera.

870. Spongiæ : Fixation.—The smaller forms (Calcispongiæ) can be fairly well fixed by the usual reagents, osmic acid being one of the best. For the larger forms no satisfactory fixing agent has yet been discovered, so far as I can ascertain. The tissues of this group are very watery, very delicate, very friable after hardening, and macerate with the greatest facility. For all but very small specimens, absolute alcohol is apparently the best fixing agent. If any watery fluid be preferred, care should at all events be taken to get the sponges into strong alcohol as soon as possible after fixation, on account of the rapidity with which maceration sets in in watery fluids. FIEDLER (*Zeit. f. wiss. Zool.*, xlvii, 1888, p. 87) has been using (for *Spongilla*), besides absolute alcohol, an alcoholic sublimate solution, and the liquids of Kleinenberg and Flemming with good effect.

Staining.—On account of the great tendency to maceration above referred to, I hold (notwithstanding many recommendations of watery stains that are to be found in the literature of the subject) that alcoholic stains be alone employed for staining sponges, and I particularly recommend Mayer's tincture of cochineal as giving the best results personally known to me. VON LENDENFELD (*Zeit. f. wiss. Mik.*, xi, 1, 1894, p. 22) uses aqueous solutions of Congo red and anilin blue for the coloration of collar-cells.

Sectioning.—Calcareous sponges may be decalcified in alcohol slightly acidified with hydrochloric acid, and then imbedded in the usual way. Siliceous sponges may be desilicified by Mayer's hydrofluoric acid method, see § 588.

Preparation of Hard Parts.—Siliceous spicules are easily cleaned for mounting by treating them on a slide with hot concentrated nitric or hydrochloric acid, or solution of potash or soda. The acids mentioned are very efficient, but it must be pointed out that they will attack the silex of some delicate spicules. Thus DEZSÖ found that the small stellate spicules of the cortex of *Tethya lyncurium* are completely dissolved by boiling hydrochloric acid. Potash solution is therefore frequently to be preferred, notwithstanding that, in my experience, it does not give such clean preparations.

According to NOLL, eau de Javelle is preferable to any of these reagents (see § 569).

Impregnation with Silver (see § 363).

Larvæ of Spongiæ.—SCHULTZE (*Zeit. f. wiss. Zool.*, xxxi, p. 295) places the ova and larvæ of *Sycandra raphanus* in hanging-drop moist chambers, oxygenated by means of a few fronds of green algæ. He also (*ibid*, xxxiv, 1880, p. 416) found that the best sections of the more advanced *sessile* larvæ of *Plakina* were obtained by selecting larvæ that had settled down on thin fronds of algæ, and treating them, together with the fronds, with osmic acid, staining with alum-carmine, and bringing into paraffin in the usual way.

Protozoa.

871. Introductory.—Since the Protozoa may be considered as free cells, and their peculiar organs known as "nucleus" and "nucleolus," "macronucleus" and "micronucleus," &c., present in the main the same reactions as cell-nuclei, it is evident that the reagents and methods of cytology are in great part applicable to this group. One of the most generally useful of these reagents will be found in the acid solution of *methyl green;* it is the reagent that allows of the readiest and best demonstration of the presence and form of the nucleus and nucleolus (BALBIANI et HENNEGUY, *Compt. rend. Soc. de Biol.*, 1881, p. 131).

Amongst useful reagents not mentioned in the following descriptions of the methods employed by different authors, I call attention to the weak solutions of alum, potash, and borax, which serve to demonstrate the striations of the cuticle and the insertions of the cilia of Infusoria.

872. Methods for quieting Infusoria.—The narcotisation methods, §§ 18 to 22, are available for this purpose.

According to SCHÜRMAYER (*Jen. Zeit.*, xxiv, 1890, pp. 402—470; *Zeit. f. wiss. Mik.*, vii, 4, 1891, p. 493) nitrate of strychnin in weak solution, 0·01 per cent. or less, gives good results with some forms, amongst which are *Stentor* and *Carchesium.* Antipyrin in concentrated solution (0·1 per cent.) or cocain of 0·01 per cent. seems only to have given good results as regards the extension of the stalk in stalked forms.

EISMOND (*Zool. Anz.*, No. 352, Dec., 1890, p. 723) has proposed a mechanical means of slowing the movements of small organisms (small worms and crustacea as well as Ciliata). He directs that a drop of thick aqueous solution of cherry-tree gum be added to the water containing the organisms (gum arabic and the like, it is stated, will not do). The objects remain fixed in their places, with cilia actively moving, and all vital processes retaining their full activity. I am greatly obliged to Dr. GRÜBLER for having been at much pains in making inquiry for me concerning the cherry-tree gum that should be used. It appears that this gum is a somewhat insoluble one, and it is difficult to get hold of a sample that will give a good solution. Further, the solutions will not keep, and must be made up fresh every day. In the face of these difficulties it would seem that the method is at present a far from perfect one. It should, however, be stated that CERTES (*Bull. Soc. Zool. France*, xvi, 1891, p. 93; *Journ. Roy. Mic. Soc.*, 1891, p. 828) has found that the method gives excellent results. He has also found that an *intra vitam* stain may be obtained by adding methyl blue or "violet dahlia No. 170," to the gum solution.

A similar process of inhibiting movements whilst preserving life has been worked out by JENSEN (after STAHL; see *Biol. Centralb.*, xii, 1892, 18, 19, p. 556: *Zeit. f. wiss. Mik.*, ix, 4, 1893, p. 483; *Journ. Roy. Mic. Soc.*, 1892, p. 891). A solution of 3 grms. of gelatin in 100 c.c. of ordinary water is made by the aid of heat. This makes a jelly at the normal temperature. It is slightly warmed, and a drop of it is mixed in a watch-glass with a drop of water containing the organisms. This plan is said to afford great facilities for the vivisection of Infusoria.

873. Staining *intra vitam.*—The possibility of staining Infusoria *intra vitam* was discovered independently and almost simultaneously by BRANDT (*Verh. d. physiol. Ges. Berlin*, 1878), by CERTES (*Soc. Zool.*, 25 janv., 1881), and by HENNEGUY (*Soc. philom.*, 12 fév., 1881). See on this subject § 213.

CERTES found that living Infusoria stain, while continuing in life for a certain time, in weak solutions of cyanin, Bismarck brown, dahlia, violet 5 B, chrysoidin, nigrosin, methylen blue, malachite green, iodine green, and other tar colours, and hæmatoxylin. The solutions should be made with the liquid that constitutes the natural habitat of the organisms. They should be very weak, that is, of strengths varying between 1 : 10,000 and 1 : 100,000. For cyanin, 1 : 500,000 is strong enough.

The "nucleus" may be stained in the living organism by dahlia and malachite green. Bismarck brown only colours the "nucleus" of certain species (*Nyctotherus*, *Opalina*—HENNEGUY). The "nucleus" frequently behaves differently in allied species.

A double stain of the nucleus (green) and protoplasm (violet) may be obtained by the simultaneous employment of dahlia and malachite green.

Examination in a coloured medium in which the organisms do not stain, but show up on a coloured background in a manner that produces somewhat the effect of dark-ground illumination, is sometimes helpful. CERTES (*Bull. Soc. Zool. de France,* xiii, 1888, p. 230) recommends solution of anilin black for this purpose; Infusoria will live in it for weeks. FABRE-DOMERGUE (*Ann. de Micrògr.,* ii, 1889, p. 545; *Journ. Roy. Mic. Soc.,* 1889, p. 832) recommends concentrated solution of diphenylamin blue.

874. Fixing and Preserving.—PFITZNER (*Morph. Jahrb.,* xi, 1885, p. 454) used concentrated solution of picric acid *run in under the cover.*

BLANC (*Zool. Anz.,* 1882, p. 22) advises liquid of Kleinenberg diluted with about a volume of water, and acidified with acetic acid.

KORSCHELT (*Zool. Anz.,* 1882, p. 217) recommends 1 per cent. osmic acid, or for Amœbæ, 2 per cent. chromic acid.

LANSBERG (*ibid.,* p. 336) advises the same reagents, but recommends *bringing the organisms into the fixing liquid with a pipette,* instead of running in the fixing liquid under the cover.

SAVILLE KENT and BERTHOLD (*Manual of the Infusoria; Journ Roy. Mic. Soc.,* 1883, p. 451) prefer a brownish-yellow solution of potassium iodide to osmic acid for fixing. See § 73.

The employment of *vapour* of iodine has been described, § 73.

CATTANEO (*Bollettino Scientifico,* iii and iv; *Journ. Roy. Mic. Soc.,* 1885, p. 538) recommends fixing for a few minutes with $\frac{1}{3}$ per cent. aqueous solution of chloride of palladium. This is said to be the best fixing agent, as it hardens in a few minutes without blackening the structures.

875. Methods of BRASS (*Zeit. f. wiss. Mik.,* i, 1884, p. 39).—He employs for fixing unicellular organisms the following liquid:

Chromic acid	1 part.
Platinum chloride	1 ,,
Acetic acid	1 ,,
Water	400 to 1000 parts.

For protozoa that are opaque through accumulation of nutritive material, he proceeds as follows:—The organisms are treated for three or four minutes with liquid of Kleinenberg, and then for some time with boiling water. They are then brought into water containing a small proportion of ammonia, in which they reassume their natural forms and dimensions. The ammonia is then neutralised by addition of a little acetic acid, and the preparation is stained with borax-carmine or ammonia-carmine. After washing, the objects are mounted in dilute glycerin. This treatment is said to afford extremely transparent preparations.

Brass also obtained good results with sublimate solution.

876. CERTES (*Comptes rend.*, 1879, 1 sem., p. 433) makes permanent preparations as follows:—Fix with osmic acid of 2 per cent. (In the case of very contractile Infusoria, place a drop of the solution *on the cover-glass*, and place it on the drop of water that contains them. But generally speaking it is best to employ only the vapour of the solution, exposing the organisms to its action for not more than from ten to thirty minutes.)

The objects having been covered, the excess of liquid is removed by means of blotting-paper, and the following stain is allowed to flow in:

Glycerin	1 part.
Water	1 ,,
Picro-carmine	1 ,,

(Eosin may also be used. Soluble anilin-blue does not give such good results.) The stain should be placed at the edge of the cover, and the slide put away in a moist chamber, in order that the water may evaporate very slowly and be changed very gradually for the glycerin mixture; if this precaution is not taken, shrinkage may occur. When the exchange has taken place, strong glycerin may be added, and gradually substituted for the dilute glycerin.

Certes states that the organisms thus prepared are fixed perfectly in their natural form, and allow of the study of the minutest detail of cilia, flagella, and the like, with the highest powers; the green coloration of Euglenæ and Paramecia is preserved. The nuclear structures are sharply brought out by the picro-carmine.

877. The Method of GÉZA ENTZ (*Zool. Anz.*, No. 96, 1881, p. 575).—A few drops of liquid of Kleinenberg are *added to a* *watch-glass of water* containing the organisms. After one or two minutes, the liquid is drawn off and the preparation is washed for half an hour with alcohol of medium strength. The objects are then stained for ten to twenty minutes in picro-carmine, washed with water till the picric acid is removed, and mounted in a mixture of equal parts of glycerin and water.

878. Other General Methods.—DU PLESSIS (VOGT et YUNG, *Trait. Anat. Comp. Prat.*, p. 92) recommends fixation with 0·2 per cent. solution of corrosive sublimate. Let the preparation *dry up*, and if the organisms have preserved their shape, stain and mount in balsam. This seemingly barbarous mode of procedure is said to give very fine preparations when successful.

FOL (*Lehrb.*, p. 102) fixes delicate marine Infusoria (*Tintinnodea*) with the perchloride of iron solution (§ 67), added to the water containing them, and stains with gallic acid as directed (§ 379), and states that this is the only method that has given him good results, especially as regards the preservation of cilia.

See also the methods of FABRE-DOMERGUE, *Ann. de Microgr.*, ii, 1889, p. 545; SCHEWIAKOFF, *Biblioth. Zool.*, v, 1889, p. 5; *Journ. Roy. Mic. Soc.*, 1889, pp. 832, 833; ZOJA, *Boll. Sci. Pavia*, 1892; *Zeit. f. wiss Mik.*, ix, 4, 1893, p. 485; LONGHI, *Bull. Mus. Zool. Univ. Genova*, 4, 1892; *Zeit. f. wiss. Mik.*, ix, 4, 1893, p. 483; BRANDT, *Fauna u. Flora d. Golfes v. Neapel*, 1885; *Journ. Roy. Mic. Soc.*, 1888, p. 665 (for Sphærozoa).

879. Demonstration of Cilia (WADDINGTON, *Journ. Roy. Mic. Soc.*, 1883, p. 185).—Solution of tannin, or a trace of alcoholic solution of sulphurous acid.

880. Stains for Flagella.—The celebrated method of LÖFFLER has run through several forms (*Centralb. f. Bacteriol.*, vi, 1889, p. 209; vii, 1890, p. 625; *Zeit. f. wiss. Mik.*, vi, 3, 1889, p. 359; vii, 3, 1890, p. 368; *Journ. Roy. Mic. Soc.*, 1889, p. 711; 1890, p. 678), of which that given here is the latest. To 10 c.c. of a 20 per cent. solution of tannin are added 5 c.c. of cold saturated solution of ferrous sulphate and 1 c.c. of (either aqueous or alcoholic) solution of fuchsin, methyl violet, or "Wollschwarz." The mixture will require for some forms the addition of a few drops of 1 per cent. solution of caustic soda; *e.g.* for typhoid bacilli, 1 c.c.; for *Bacillus subtilis*, 28 to 30 drops; for bacilli of malignant œdema, 36 to 37 drops. Some other forms will require besides the addition of a trace of sulphuric acid to the soda solution: so for cholera bacteria, half a drop to 1 drop; for *Spirillum rubrum*, 9 drops.

Cover-glass preparations are made and fixed in a flame in the usual way, special care being taken not to over-heat. Whilst still warm the preparation is treated with mordant (*i. e.* the above-described mixture), and is heated for half a minute until the liquid begins to vaporise, after which it is washed in distilled water and then in alcohol. It is then treated in a similar manner with the stain, which consists of a saturated solution of fuchsin in anilin water, the solution being preferably neutralised to the point of precipitation by cautious addition of 0·1 per cent. soda solution.

The modifications of this method by Bunge are as follows:—Firstly (see *Journ. Roy. Mic. Soc.*, 1894, p. 640, or *Zeit. f. wiss. Mik.*, xiii, 1, 1896, p. 96), he modifies the mordant by taking *Liquor Ferri Sesquichlorati* instead of the sulphate. He dilutes the Liquor Ferri Sesquichlor. with twenty vols. of distilled water, takes three parts of the tannin solution and one part of the dilute iron solution, and adds to 10 c.c. of the mixture 1 c.c. of saturated aqueous solution of fuchsin. The mordant should be allowed to ripen exposed to the air for some days or weeks. In a later paper (see *Journ. Roy. Mic. Soc.*, 1895, pp. 129 and 248) he recommends adding to the ripened mordant a few drops of peroxide of hydrogen, until it becomes of a red-brown hue. It is then shaken up and filtered on to the prepared cover-glass, on which it is allowed to act for about a minute. The cover-glass is then mopped up, dried, and stained, preferably with carbol-gentian.

Trenkmann (*Centralb.*, vi, 1889, p. 433; *Zeit. f. wiss. Mik.*, vii, 1, 1890, p. 79) mordants for several hours at the normal temperature in a 1 per cent. solution of tannin in 0·5 per cent. hydrochloric acid, and stains for several hours in carbolic fuchsin; and gives also two other similar methods.

Brown (*The Observer*, iii, 1892, p. 298; *Journ. Roy. Mic. Soc.*, 1893, p. 268) mordants for several hours in a mixture of 30 gr. tannin, 12 drops anilin oil, and 1 fl. oz. of alcohol, which may, if required, be alkalised by addition of a trace of caustic soda (so for *Spirillum undula* and *Bacillus ulna*), or be acidified for some forms with a little hydrochloric acid. The cover is stained by the process of heating over a flame for a few minutes with any anilin-water solution of fuchsin, methyl violet, dahlia, methyl green, &c., neutralised with caustic soda as in Loeffler's process, or with a solution of rosanilin in anilin water.

See also Julien, quoted *ibid.*, 1894, p. 403; van Ermengem, *ibid.*, p. 405; Sclavo, quoted *Zeit. f. wiss. Mik.*, xiii, 1, 1896, p. 96; and Hessert, *ibid.*, p. 98.

APPENDIX.

881. The Usual Alcohols.—The following, or a similarly-spaced series of alcohols, should be kept on the table.

Absolute Alcohol.—See § 84. The so-called "absolute alcohol" of commerce is generally of about 98 per cent. strength. This grade is convenient, but *not necessary* for ordinary work.

95 per cent. Alcohol.—This is the average strength of the common strong commercial alcohol, which ranges in general from 94 per cent. to 96 per cent. according to temperature. The strength of this, or of the following, should be determined by means of an areometer (Gay Lussac's being very convenient), so as to form a starting-point for the following mixtures, which may be made by means of the subjoined table. This is the *usual grade for dehydrating* before clearing. It is the highest grade that should be used for dehydrating celloidin sections.

90 per cent. Alcohol.—May be made by taking 100 vols. 95 per cent. alcohol, and 5·5 vols. water. This is the usual strength of the strongest commercial *Methylated Spirit,* which (if free from mineral naphtha) may be taken instead of pure alcohol for common work. If naphtha be present the alcohol becomes turbid on the addition of water. Oil of bergamot will clear from this grade.

85 per cent. Alcohol.—Rectified Spirit, B.P., is a little weaker than this, viz. 84·5 per cent.

70 per cent. Alcohol.—Only exceptionally powerful clearers, such as anilin oil, will clear from this grade; see § 125. *This is the proper grade in general for preserving organisms and tissues in* (but see the remarks on pp. 4 and 5); higher grades should not generally be used unless it is desired to harden. *This is the proper grade for washing out borax-carmine stains, corrosive sublimate after fixing,* &c.

50 per cent. Alcohol.—This is the strength of *Proof Spirit.*

"One-third Alcohol."—Made by taking 1 vol. of 90 per cent. alcohol, and 2 vols. water. See § 83.

882. Table for diluting Alcohol (after GAY-LUSSAC).—To use this table, find in the upper horizontal row of figures the percentage of the alcohol that it is desired to dilute, and in the vertical row to the left the percentage of the alcohol it is desired to arrive at. Then follow out the vertical and horizontal rows headed respectively by these figures, and the figure printed at the point of intersection of the two rows will show how many volumes of water must be taken to reduce *one hundred volumes* of the original alcohol to the required grade. Thus, if it be required to manufacture some 70 per cent. alcohol, starting with 90 per cent., we find the figure 90 in the upper column, the figure 70 in the vertical column, and at the point of intersection we read 31·05, showing that a fraction more than 31 volumes of water must be added to 100 volumes of 90 per cent. alcohol. Or similarly, if we wish as before to make 70 per cent. alcohol, but start with an alcohol of 85 per cent., we find that 23·14 volumes of water must be employed.

Weaker grade required.	ORIGINAL GRADE.								
	90 p. 100.	**85** p. 100.	**80** p. 100.	**75** p. 100.	**70** p. 100.	**65** p. 100.	**60** p. 100.	**55** p. 100.	**50** p. 100.
p. 100. **85**	6·56								
80	13·79	6·83							
75	21·89	14·48	7·20						
70	31·05	23·14	15·35	7·64					
65	41·53	33·03	24·66	16·37	8·15				
60	53·65	44·48	35·44	26·47	17·58	8·76			
55	67·87	57·90	48·07	38·32	28·63	19·02	9·47		
50	84·71	73·90	63·04	52·43	41·73	31·25	20·47	10·35	
45	105·34	93·30	81·38	69·54	57·78	46·09	34·46	22·90	11·41
40	130·80	117·34	104·01	90·76	77·58	64·48	51·43	38·46	25·55
35	163·28	148·01	132·88	117·82	102·84	87·93	73·08	58·31	43·59
30	206·22	188·57	171·05	153·61	136·04	118·94	101·71	84·54	67·45

883. Histological Reagents and Apparatus.—See § 216. As regards the products of Grübler and Co., so often quoted in the foregoing pages, I would add that they should either be ordered from them direct, or, if ordered through any agent, should be ordered to be sent in the original packages, signed *and dated* by them. This is in order to ensure the due *freshness* of the products; many of them will not keep well for very long. Glass and other apparatus can be obtained as well as chemicals from the above-quoted houses.

Want of space compels me to suppress the lists of suggested reagents given in a former edition under the headings "The Laboratory Table" and "The Zoologist's Travelling Case." Either collection may still be obtained from Grübler and Co., the latter in appropriate bottles, fitted into a case measuring 1 foot 4 inches × 5½ inches × 4½ inches, at the price of about £2 5*s*., or a case somewhat larger, yet not too heavy to be carried in the hand, at about £3.

884. Cleaning Slides and Covers.—The readiest way known to me of freeing slides from balsam, damar, and cement is to wet with water and scrape with an old knife, using afterwards, if necessary, one of the solvents mentioned below.

Hanaman, *Journ. Roy. Mic. Soc.*, i, 1878, p. 295; *American Naturalist*, xii, p. 573.—To a cold saturated solution of bichromate of potash add ⅛ of its bulk of strong sulphuric acid (care must be taken on account of the heat and vapours evolved).

Heneage Gibbes, *ibid.*, iii, 1880, p. 392.—Place the cover-glasses in strong sulphuric acid for an hour or two, wash well until the drainings give no acid reaction; wash first with methylated spirit, and then with absolute alcohol, and wipe carefully with an old silk handkerchief.

Seiler, *ibid.*, p. 508.—*New* slides and covers are placed for a few hours in the following solution:

Bichromate of potash	3 ounces.
Sulphuric acid	3 fluid ounces.
Water	25 „

Wash with water. The slides may be simply drained dry; the covers may be wiped dry with a linen rag.

Slides and covers that have been used for mounting either with balsam or a water medium are treated as follows:—The covers are pushed into a mixture of equal parts of alcohol and hydrochloric acid, and after a few days are put into the bichromate solution and treated like new ones. The slides are scraped free of the mounting medium with a knife and put directly into the bichromate solution.

Fol (*Lehrb.*, p. 132) recommends either a solution containing 3 parts of bichromate, 3 of sulphuric acid, and 40 of water; or simply dilute nitric acid.

Garbini (*Manuale*, p. 31) puts slides for a day into 10 per cent. sulphuric acid, then washes, first with water and then with alcohol.

Behrens (*Zeit. f. wiss. Mik.*, 1885, p. 55) treats slides first with concentrated nitric acid, then with water, alcohol, and ether.

James (*Journ. Roy. Mic. Soc.*, 1886, p. 548) treats used slides with a mixture of equal parts of benzin, spirit of turpentine, and alcohol.

Knauer (*Centralbl. f. Bakt.*, x, 1891, p. 8; *Zeit. f. wiss. Mik.*, ix, 2, 1892, p. 187; *Journ. Roy. Mic. Soc.*, 1891, p. 833) recommends boiling for twenty or thirty minutes in 10 per cent. lysol solution, then rinsing with cold tap water till clear.

Nias (*Journ.*, pag. cit.) finds it is sufficient to boil with washing soda, and rinse.

In the employment of the water or alcohol section-fixing method, § 186, it is extremely important to work with slides absolutely free from grease. After the slides have been cleaned by one of the processes given above, they should be rinsed with distilled water and preserved in 90 per cent. alcohol, from which they should be removed with forceps when required for use, not with the fingers, then simply drained, or wiped with a very clean cloth.

885. Gum Mucilage for Labels, &c.—The *Journ. of the Chemical Soc.* says that the adhesive qualities of gum may be very much exalted by the addition of aluminium sulphate (the so-called "patent" alum) to the mucilage. "2 grms. of crystallised aluminium sulphate, dissolved in 20 grms. of water, is added to 250 grms. strong gum arabic solution (2 grms. in 5 grms. water). Ordinary solutions of gum arabic, however concentrated, fail in their adhesive power in many cases, such as the joining together of wood, glass, or porcelain; prepared, however, according to the above receipt, the solution meets all requirements" (from *Public Opinion*, Feb. 19th, 1886).

Fol (*Lehrb.*, p. 148) advises that slides be prepared for labelling by spreading over one end a layer of aluminium-chloride gelatin dissolved in acetic acid, and allowing it to dry before putting on the label.

Why do not the glass makers furnish slides with roughened (ground) end-surfaces for the reception of labels?

For four other receipts for gums and pastes for labels, see Eliel, in *Engl. Mechan.*, 1887, p. 535; *Amer. Mon. Mic. Journ.*, 1887, p. 93; *Zeit. f. wiss. Mik.*, v, 1, 1888, p. 69.

Vosseler (*Zeit. f. wiss. Mik.*, vii, 4, 1891, p. 459) recommends, for attaching protective cardboard ridges to slides, a syrup-thick solution of bleached shellac in alcohol.

886. Green Light.—The suggestion of the employment of green light in microscopy is, I believe, due to Englemann (*Pflüger's Arch.*, 1880, p. 550). He strongly recommends the use of green light for delicate observations, as giving sharper definition, allowing finer details to be seen, and tiring the eyes less than white light. Green glass of sufficiently good quality is found in commerce. The glass is best put between the mirror and the object, *e. g.* on the diaphragm. Blue glass (cobalt or ammonio-sulphate of copper) is also useful, but less so than green. Red light is most hurtful. " The explanation of these points, so important in practice, may be found in the results obtained by Lamansky in his researches on the 'Limits of Sensibility of the Eye to the Different Colours of the Spectrum' *Arch. f. Ophthalm.*, xvii, p. 123, 1871)." I would add that for lamp-light work, especially fine work with high powers, either green or blue glass is, according to my experience, a *sine quâ non* if the best attainable results be desired. I always use blue *cover-glasses*, putting from one to four of them on the diaphragm of the condenser. For some unexplained reason I find I get better results by means of several superposed thin glasses than by one thick one.

887. Re-staining Old Mounts (HENNEGUY, from the last edition of the *Traité des Méthodes techniques de l'anat. microscopique*, LEE et HENNEGUY). It is probably not generally known that balsam mounts the stain of which has faded, or which it may be desired to submit to some other staining process or mount in some other medium, may often with great advantage be re-stained and re-mounted. All that is necessary is to put the slide into a tube of xylol or benzol till the cover falls off (about two days), wash well for some hours in clean xylol, and pass through alcohol into the new stain. Since this was pointed out to me by Dr. Henneguy, I have unmounted and re-stained a considerable number of old preparations, some of them over fifteen years old, and have been most agreeably surprised at the results obtained. I have succeeded in every case with series of sections mounted on Mayer's albumen, but I doubt whether the process would be safe with sections mounted on such a fixative as Schällibaum's collodion or the like.

INDEX.

*The numbers refer to the Pages.**

A.

ABBE, mounting medium, 277.
Absolute alcohol, uses, 4, 5; preparation, 52.
Acanthocephali, 480.
Acephala, *see* Mollusca.
Acetate of alumina, for mounting, 265.
Acetate of copper, 46, 267.
Acetate of lead, 61, 392.
Acetate of potash, 265.
Acetate of uranium, 46.
Acetic alcohol, 45.
Acetic acid for fixing, 44, 45, 467.
Aceto-carmine, 152, 155.
Acetone, for dehydration, 4.
Acid anilin dyes, 179.
Acid, free, test for, in tissue, *see* Congo red.
Acid fuchsin, 219; for nerve tissue, 403, 414.
Acid Magenta, *see* Acid fuchsin.
Acidophilous cell-elements, 446.
Acids, *see* Acetic, Chromic, Formic, Hydrochloric, &c.
Acidulated alcohol, 52.
Actiniaria, 13—17, 488.
ADAMKIEWICS, stain for nerve-centres, 414.
Adipose tissue, 35, 445.
Adjective staining, 140, 141, 224.
Agar-agar, section fixative, 127.
AGASSIZ and WHITMAN, pelagic ova, 346.
Albumen, imbedding mass, 118; injection mass, 307; section-fixing process, 123, 126; removal of, from ova, 342 *et seq.*
Albuminoids, reactions, 357.
Alcohol, its action on tissues, 5; fixing, 51; absolute, 52; "one third," 51; acetic, 45; acidulated, 52; picric, 50; for hardening, 62; macerating, 313; preserving tissues, 4, 5, 266; the usual grades, 500; table for diluting, 501.
Alcohol balsam, 280.
Alcoholic carmines, 157, 158.
Alcoholic cochineal, 158, 159.
Alcoholic hæmatein stains, 172—174.
Alcyonaria, 489.
Alcyonella, 14.
Alcyonium, 12, 489.
Aldehyde green, 198.
ALFEROW, silver impregnation, 247.
Alizarin, artificial, 232.
ALLEGER, serial sections, 127.
ALLEN, methylen blue, 207.
ALT, Congo red nerve stain, 418.
ALTMANN, fixing methods, 35, 364; paraffin stove, 82; bioblasts, 138, 364, 368; impregnation and corrosion methods, 323.
Alum for fixing, 37; ammonia-alum, solubility in water, 170.
Alum-carmine, 150—152; ditto with acetic acid, 151; with picric acid, 152.
Alum-cochineal, 150.
Alumina acetate, 265.
Amarœcium, 346.
Amber varnish, 289.
Ammonia bichromate, 60, 392; neutral chromate, 61.

---

* In accordance with a wish expressed by readers in whose judgment I have confidence, the references are now given to the pages, and not as heretofore to the numbered sections.

The numbers refer to the Pages.

Ammonia-alum, 170.
Ammonia-carmine, 155, 415.
Ammonium molybdate, for impregnation, 261; for fixing nuclei, 364; sulphocyanide, 315; vanadate, 310.
Amphibia, embryology, 342 *et seq.*
Amphioxus, 378.
Amphipoda, embryology, 352.
Amphiura, 488.
Amphophilous cell-elements, 446.
Amyl nitrite, 293.
Amyloid matter, 199, 201.
Anchinia, 467.
Ancylus, 469.
ANDEER, phloroglucin, 326.
ANDRÉ, fixing *Ancylus*, 469.
ANDRES, Actiniæ, 13, 14, 488; section-stretcher, 87.
ANDREWS, imbedding apparatus, 77.
Anilin, for clearing, 70, 109.
Anilin dyes, generalities, 179 *et seq.*; classification into acid and basic, 179; regressive staining process, 182 *et seq.*
Anilin black, 231, 475.
Anilin blue, 230.
Anilin blue-black, 231, 416.
Anilin green, 228.
Anilin oil for clearing, 70, 109; for staining, 190.
Anilin red, 196.
Anilin violet, 200.
Anilin water, 190.
Aniseed imbedding mass, 118.
Annelida, 483—485; embryology, 352.
Anodonta, 15.
Antedon, 488.
Anthea, 488.
Anthozoa, 488.
Antipathes, 489.
Antipyrin for narcotisation, 495.
APÁTHY, celloidin imbedding, 100; preserving the blocks, 106; orientation of objects, 103; serial sections, 129, 130; cement, 289; hæmatoxylin stain, 174; methylen-blue impregnation, 202, 205, 207—210; mounting medium, 269.
APEL, *Sipunculus* and *Halicryptus*, 481.
Aphides, preparation and embryology, 351.
Aplysia, 468.
Aqua Javelli, 322, 330.
Arabin, 126.
Araneina, embryology, 351.
ARCANGELI, carmines, 155.
Archil, 234.
Archiplasm, archoplasm, *see* Cytological methods, Plasma stains.
Arctiscoida, 475.
Areolar tissue, 444.
ARNOLD, critique of Flemming's mixture, 32, 34.
ARNSTEIN, methylen-blue method, 206, 209, 210; gold method, 374.
ARONSON, gallein stain, 436.
Arsenic acid for decalcification, 326.
Arthropoda, general methods, 472; clearing and softening chitin, 472; depigmentation, 473; eyes, 473—475; nerve and muscle, 475; embryology, 348—352.
Artificial alizarin, 232.
Artificial fecundation, 331.
Artificial œdemata, 444.
Artificial pigment spots, 59, 391, 392.
Artificial saliva, 315.
Artificial serums, 264, 265.
Ascaris, ova, 45, 368.
Ascidians, 16, 346, 467.
Asphalt varnish, 286.
Asphyxiation as a killing method, 16.
ASPINALL'S enamel, for cement, 290.
Astacus, embryology, 352.
Asteracanthion, 14.
Asteroidea, 486.
Astroides, 489.
Attraction spheres, *see* Cytological methods, Plasma stains.
AUBERT, cements, 285.
AUGSTEIN, *Strongylus*, 480.
Aurantia, 229.
Auréoline, 329.
Auricularia, 487.
Aves, embryology, 338 *et seq.*
Axis cylinder, 403 *et seq.*; stains, 415 *et seq.*
Axolotl, ova, 343.
AZOULAY, nerve-impregnation, 412.

The numbers refer to the Pages.

B.

Baber, picro-carmine, 154.

Babes, safranin stain, 190; supersaturated do., 365.

Balbiani, "noyau vitellin," 368; living ova, 332; ova of Phalangida, 351; Protozoa, 495.

Balfour, embryology of Araneina, 351.

Ballowitz, muscle of Cephalopoda, 383.

Balsam, 277 *et seq.*

Balzer, elastic tissue, 450.

Barff, boroglyceride, 272.

Barnes, observation of Trichinæ, 480.

Barrois, embryology of Echinodermata, 487.

Basic anilin dyes, 179.

Basophilous cell-elements, 446.

Bastian, gold method, 255.

Bataillon and Koehler, borax methylen blue, 365.

Baumgarten, fuchsin and methylen-blue stain, 232; bleu de Lyon, 237.

Bayerl, stain for cartilage, 236, 455; decalcification, 325.

Beale, digestion fluid, 320; glycerin jelly, 273; injections, 308; varnish, 289.

Bechtereff, hardening brain, 392.

Beck, cements, 285.

Becker, microtome, 72.

Bedot, fixing process, 492.

Behn, hardening skin, 370.

Behrens, cements, 285; indulin and nigrosin, 228; amber varnish, 289; cleaning slides, 503.

Béla Haller, macerating mixture, 318.

Bellarminow, injection, 310.

Bellonci, preparation of brain, 413.

Bell's cement, 286.

Benda, iron hæmatoxylin, 176; copper hæmatoxylin, 177; double-stain, 227; freezing brain, 400.

Benecke, connective-tissue fibrils, 444.

Bengal rose, 226.

Benzin, for imbedding, 79.

Benzol, for clearing, 70.

Benzo-azurin, 197.

Benzo-purpurin, 225.

Benzoyl green, 228.

Bergamot oil, 68.

Bergonzini, staining mixture for plasma cells, 449.

Berkley, cerebellum, 398; rapid Weigert stain, 411.

Berlin blue, 302, 309.

Berlinerblau, F., regeneration of Weigert's hæmatoxylin, 407.

Bernheim, gold process, 257; bladder of frog, 384.

Bernheimer, hæmatoxylin for retina, 441.

Bethe, methylen blue, 212; stain for chitin, 475.

Betz, central nervous system, 392—394.

Bevan Lewis, *see* Lewis, Bevan.

Bianco, S. Lo, chromo-acetic acid, 45; Molluscoida (Bryozoa), 468; Mollusca, 468; Nemertina, 478; Gephyrea, 481; Chætopoda, 484; Holothurioidea, 485; Medusæ, 491; Siphonophora, 493; Ctenophora, 493; Tunicata, 16, 467; narcotising mixture, 14.

Bichloride of mercury, *see* Corrosive sublimate.

Bichromate of ammonia, 60.

Bichromate of potash, for fixing, 36, 60; for hardening, 58 *et seq.*, 391 *et seq.*; for maceration, 317.

Bichromate of silver impregnation, *see* Golgi.

Bickfalvi, digestion fluid, 320.

Biebricher Scharlach, 226.

Biedermann, methylen-blue method, 207; nerve-endings in muscle, 379.

Binet, bleaching osmic objects, 28.

Biniodide of mercury medium, 275.

Bioblasts, Altmann's, 138, 364, 368.

Biondi, staining mixture, 219, 223; methods for blood, 457, 461.

Bipinnaria, 487.

Bismarck brown, for regressive staining, 196; for progressive, 200.

Bitume de Judée, 286.

Bizzozero, gentian violet, 192, 193; picro-carmine, 154; blood, 458, 460, 461.
Bjeloussow, injection, 307.
Blackley blue, 228.
Bladder of frog, 384.
Blanc, Infusoria, 497.
Blattida, embryology, 350.
Blaue, glandular epithelium, 370.
Bleaching, 328 *et seq.*; bichromate objects, 58; Erlicki objects, 59; osmic objects, 27.
Bleu alcool, 230.
Bleu carmin aqueux, 231.
Bleu de Lyon, 230.
Bleu de nuit, 230.
Bleu lumière, 230.
Blochmann, ova of Amphibia, 342; nervous system of Cestodes, 433.
Blood, 456—461.
Blue-black, 231, 416.
Blum, formaldehyde, 53, 62.
Bobretzky, embryology of Lepidoptera, 350.
Boccardi, gold method, 256; motor plates, 381.
Böhm, gold method, 256; Golgi's impregnation, 427.
Böhmer, hæmatoxylin, 170.
Böhmig, Rhabdocœla, 477.
Bone, decalcification, 323 *et seq.*; preparation, 451—455.
Borax-carmine, Grenacher's, 156; aqueous, 155; with indigo-carmine, 235, 236.
Borax-methylen blue, 365.
Bordeaux-iron-hæmatoxylin, 366.
Boric-acid carmine, 155.
Born, paraffin imbedding, 77; section-fixing, 127; reconstruction of sections, 333; section-stretcher, 87; embryological method, 344.
Boroglyceride, 272.
Böttcher-Hermann staining process, 182.
Bouma, stain for cartilage, 455.
Boveri, ovum of Ascaris, 368; medullated nerve, 404.
Boyer, embryology of Teleostea, 345.
Brachionus, 482.
Brady, chloral hydrate medium, 266.
Bradynema, 480.
Brain, *see* Central nervous system and Neurological methods.
Brandt, glycerin jelly, 273; Protozoa, 496, 499.
Brass, alcoholic carmine, 158; bleaching osmic objects, 28; paraffin imbedding, 79, 94; Protozoa, 497.
Braun, Alcyonaria, &c., 489; Rhabdocœla, 477; Nematodes, 480.
Breckenfeld, *Hydra*, 490.
Breglia, Weigert's hæmatoxylin, 407, 411.
Bremer, motor plates, 381; methylen blue and eosin, 227.
Bride, Mac-, *see* Macbride.
Bristol, regeneration of osmic acid solutions, 25.
Brock, macerating medium, 317.
Brösicke, staining method, 260.
Brown, stains for flagella, 500.
Brücke, digestion fluid, 320; injections, 302.
Brun, mounting medium, 270.
Brunotti, gelatin imbedding mass, 98.
Brunswick black, 286.
Bryozoa, 14, 15, 468.
Budge, injection, 310.
Bugula, 468.
Bumpus, celloidin imbedding, 110.
Bunge, stains for flagella, 500.
Burci, elastic tissue, 451.
Burckhardt, nervous system of *Protopterus*, 398.
Bürger, nervous system of Nemertina, 206.
Busch, eosin and hæmatoxylin, 237; decalcification, 323, 324.
Busse, photoxylin imbedding, 100; celloidin do., 101, 103, 106.
Bütschli, paraffin imbedding, 81; hæmatoxylin stains, 171, 177.

C.

Cajal, Ramón y, silver chromate impregnation, 425; terminations of nerves and tracheæ, 380; retina, 441; sympathetic ganglia, 425.

The numbers refer to the Pages.

Calberla, methyl green, 197, 199; ditto and eosin, 226; indulin, 228; glycerin mixture, 272; macerating mixture, 315.
Caldwell, serial sections, 123.
Cambridge rocking microtome, 72.
Canada balsam, for mounting, 279 *et seq.;* imbedding method, 115.
Canfield, iris, 383.
Cannel oil (cinnamon oil), 68.
Caoutchouc, for section-fixing, 128; cement, 286.
Capparelli, dichroïsm of methyl violet, 201.
Carazzi, bleaching method, 329.
Carbolic acid, for clearing, 69, 109.
Carbolic fuchsin, 196.
Carbolised syrup, 265.
Carbonic acid narcotisation method, 17.
Carchesium, 495.
Carcinoma corpuscles, 192.
Carmalum, 149.
Carmine, analysis of, 145; generalities on, 145—149; theory of staining with, 145; practice of staining, 148; for what purposes useful, 148.
Carmine blue, 231.
Carmine stains, aqueous, 149—155; alcoholic, 156—160; choice of, 148; carmine combination stains, 235 *et seq.;* and *see* the names of the respective authors, as also the separate entries, "Alum-carmine," "Borax-carmine," &c.
Carminic acid, 146.
Carminroth, 155.
Carnoy, acetic alcohol, 45; chromo-aceto-osmic acid, 34; cement, 290; cytological methods, 357, 358, 364; salt solution, 263; tannin solution, 267.
Carpenter, cements and varnishes, 284.
Carrière, corpuscles of Herbst and Grandry (gold method), 375; eyes of Gastropods, 469.
Carter, injection mass, 300.
Cartilage, 455, 456.
Caryophyllia, 489.
Cassiopeia borbonica, 491.
Castellarnau, de, Lamellibranchiata, 468; Medusæ, 491; Nemertina, 479; Ophiuridea, 486; Zoantharia, 489.
Castor oil for mounting, 282.
Cattaneo, corpuscles of Golgi, 381; Infusoria, 43, 497.
Caustic potash and soda, for maceration, 314; for corrosion, 322.
Cavazzani, triple stain, 239.
Cedar oil, for minute dissections, 9; for paraffin imbedding, 80; clearing, 66; for mounting, 278.
Cell granules, 368 *et seq.*, 445—450, 459.
Cell researches, *see* Cytological methods.
Cellepora, 468.
Celloidin, 99 *et seq.;* the new method, 110; the dry method, 111; clearing sections, 108; injection masses, 310.
Cells, paper, for mounting, 285.
Cements, 284 *et seq.;* generalities, 284; comparative tenacity of, 285.
Central nervous system, introduction, 385, 386; fixing, 387, 388; hardening, 388—398; imbedding and cutting, 398—401; nerve-fibre stains, 405—414; axis-cylinder and protoplasm stains, 415—419; impregnations, 419; dry processes for preserving brains, 437; methylen blue for, 443; neuroglia, 435, 436; myelon of reptiles, &c., 398; and *see* Nerves, Retina, &c., and the names of Authors.
Centrosomes, *see* Cytological methods, Plasma stains.
Cephalopoda, eyes, 470; ova, 346.
Cercariæ, 477.
Cerebellum, cerebrum, *see* Neurological methods.
Cerfontaine, curarising *Lumbricus*, 483.
Certes, Protozoa, 496, 497, 498.
Cestodes, 476.

The numbers refer to the Pages.

Chætopoda, 483.
Chætopteridæ, 484.
Chain section-cutting, 88.
CHEATLE, dehydration apparatus, 4.
Cherry gum, 495.
Chemicals, 143.
CHENZINSKY, methylen blue and eosin, 227.
CHICHKOFF, Turbellaria, 478.
China blue, 231, 403.
Chinolin blue, 228.
Chironomus, embryology, 351.
Chitin, clearing and softening, 472 *et seq.;* staining, 475.
Chitonidæ, embryology, 348; eyes, 470.
Chloral hydrate for narcotising, 14; preservative solutions, 265; for maceration, 314; Gilson's mounting medium, 275; Geoffroy's ditto, *ibid.*
Chlorate of potash for maceration, 318.
Chloride-of-aluminium carmine, 150.
Chloride of copper fluid, 46.
Chloride of gold, *see* Gold chloride.
Chloride of magnesium for narcotisation, 16.
Chloride of manganese examination medium, 263.
Chloride of palladium, *see* Palladium chloride.
Chloride of platinum, 36, 42, 43, 59, 61.
Chloride of vanadium, 418, 441.
Chloride of zinc, for hardening brain, 396, 397; for impregnation, 434.
Chlorine bleaching methods, 328.
Chloroform, for paraffin imbedding, 80, 81; for celloidin ditto, 104; for bleaching, 330; for narcotisation, 13; for clearing, 70.
CHOLODKOWSKY, embryology of Blattida, 350.
Chromate of ammonia, 61.
Chromate of lead stain, 261.
Chromate of silver impregnation, *see* GOLGI.
Chromates as fixing agents, 37.
Chromatin, reactions of, 357—360; solvents of, 358; stains for, 365.
Chromic acid, generalities, 29; fixing, 29; hardening, 56; killing, 22; maceration, 317; decalcification, 324, 326; chromic acid with acetic acid, 30; with alcohol, 30, 57; with nitric acid, 35; with platinum chloride, 36; with osmic acid, 31; with acetic and osmic acid, 31 *et seq.*
Chromic objects, action of light on, 30; bleaching, 30.
Chromo-acetic acid, 30; chromo-aceto-osmic, 31—35, 361; chromo-formic, 31; chromo-nitric, 35; chromo-osmic, 31, 57|; chromo-picric, 36, 57; chromo-platinic, 36.
CHRSCHTSCHONOWIC, gold method, 255.
CHUN, imbedding Siphonophora, 95.
CIACCIO, gold process, 256; motor plates, 381; corpuscles of Golgi, 382.
CIAGLINSKI, spinal cord, 414.
Cilia of Infusoria, 499.
Ciliated epithelium, 471.
Cinnamon oil, *see* Cannel oil.
Ciona, 467.
Cladocora, 489.
CLARKE'S spirit-proof cement, 286.
Clasmatocytes, 450.
Clavellina, 467.
CLAYPOLE, serial sections, 124.
Cleaning slides and covers, 503.
Clearing, generalities, 6; practice of, 64.
Clearing agents, 64 *et seq.:* STIEDA'S experiments on, 65; NEELSEN and SCHIEFFERDECKER'S, 65—68; celloidin sections, 108; paraffin ditto, 70.
Clove oil, 9, 67; for imbedding, 79.
Coal-tar chromatin stains, 180 *et seq.;* regressive ditto, 182—197; progressive ditto, 197—209; coal-tar plasma stains, 215—232.
Coal-tar colours, *see* Anilin dyes.
COBB'S differentiator, 4.
Cocain for narcotising, 15.
Coccidæ, 472.

The numbers refer to the Pages.

Cochineal, theory of staining with, 147; use of, 159; HERRICK'S 151; CZOKOR'S, 150; KLEIN'S, 151; MAYER'S, 158, 159; PARTSCH'S, 150.
Cochlea, 442, 443.
Cœlenterata, 488—493.
COHNHEIM, gold staining, 252; silver staining, 668.
COLE, freezing method, 116.
Collagen, 444 *et seq.*
COLLIN, *Criodrilus*, 483.
COLLINGE, pelagic fish ova, 346.
Collodion imbedding, 99 *et seq.;* section-fixing process, 120—122.
Collodionising paraffin sections, 89.
Colophonium, cement, 288; imbedding mass, 115; mounting medium, 281.
COLUCCI, retina, 441.
Comatula larvæ, 488.
Combination stains, 233 *et seq.;* and *see* Stains.
Congelation imbedding methods, 115—118.
Congo red, 225. 402, 414.
Conjunctiva, 375.
Connective tissue, 444 *et seq.*
CONTEJEAN, Ranvier's hæmatoxylin, 173.
Convoluta, 477.
Copal, imbedding process, 114; varnish for mounting, 282.
Copepoda, 472.
Copilia, 472.
Copper chloride and acetate preservative mixture, 267.
Copper sulphate fluids, fixing, 344, 492, 493; impregnation process, 434; hæmatoxylin stain, 177.
Coral, 489.
Corallin, 196.
CORI, narcotisation, 14, 15; Flemming's mixture, 33; preserving osmic acid solutions, 25.
Cornea, 375—377.
Corpuscles, tactile, 373 *et seq.;* corpuscles of Golgi, 381, 382; of Herbst and Grandry, 374; of Krause, 375; of Meissner, 375.
Corrosion, 322 *et seq.;* ALTMANN'S methods, 323.
Corrosive sublimate, 38; fixing liquids, 38—42; preservative liquids, 266.
Cotton blue, 231.
COUPIER'S blue, 228.
Covers and slides, cleaning, 503.
COX, Golgi's mercuric impregnation, 434.
Creosote, *see* Kreasote.
Creseis, 469.
Criodrilus, 483.
Cristatella, 14, 468.
Crocein, 219.
Crystal violet, 372.
Crystalline, 315, 377.
Ctenophora, 493.
CUCCATI, picro-carmine, 154; hæmatoxylin, 173; retina, 441; soda-carmine, 155.
Cucumaria, 486.
Cupric fixing mixtures, 492, 493; preservative ditto, 267; impregnation process, 434.
Curare for narcotisation, 16, 354.
CURSCHMANN, amyloid matter, 199.
Cyanide of mercury, for fixing, 481.
Cyanin, 228.
CYBULSKY, gold method, 258.
Cynthia, 467.
Cytological methods, 354 *et seq.;* subjects for study, 354—356; observation of living cells, 354; staining ditto, 356; fresh cells, 356; micro-chemical reactions, 357—360; fixing, 360—365; cytological stains, 365—368; Nebenkern, Archoplasmakugel, sphère attractive, *ibid.;* nucleus of Balbiani, 368; cell granules, 368.
CZERNY, macerating mixture, 316.
CZOKOR, cochineal, 150; turpentine cement, 287.

D.

Dahlia, 195.
Dammar, 280.
DASZKIEWICZ, KORYBUTT-, plasma-cells, 446.

The numbers refer to the Pages.

Davidoff, ova of *Distaplia*, 346.
Davies, injection, 300.
Deane, mounting medium, 268; glycerin jelly, 273.
Decalcification, 323 *et seq.*
Decapoda, 472.
Decker, section stretcher, 87.
Deecke, hardening brain, 396; imbedding and cutting, 400.
Definition, affected by transparency of stains, 188; green light for, 504.
Degenerate nerves, 412.
Dehydration, 3, 4.
Dejerine, neurological methods, 386, 400.
Dekhuysen, fat, 445; blood, 459; silver staining, 246.
Delafield, hæmatoxylin, 170.
Delage, osmium-carmine, 155; Rhabdocœla, 477.
Deltapurpurin, 225.
Dendrocœla, 478.
Dendrophyllia, 489.
Dendy, *Geonemertes*, 479.
Depigmentation, 328 *et seq.*, 472 *et seq.*
De Quervain, fixation of nerve-centres, 387.
Desilicification, 327.
De Souza, pyridin, 62.
Dextrin freezing mass, 117.
Dezsö, *Tethya*, 494.
Differentiators, 4.
Diffusion apparatus, 4.
Diffusion currents, to avoid, 4.
Digestion, 320 *et seq.*, 358.
Dimmock, carminic acid, 145, 155.
Dinitrosoresorcin, 403.
Diomidoff, hardening brain, 392.
Diphyes, 493.
Diptera, embryology, 351.
Dissections, minute, 6, 9.
Dissociation, 312 *et seq.*
Distaplia, 346.
Dogiel, olfactive organs, 375; iris, 383; methylen-blue impregnation method, 207, 209, 212; corpuscles of Herbst and Grandry, 374; of Meissner, 375; retina, 441.
Domergue Fabre, *see* **Fabre Domergue**.
Donaldson, hardening brain, 391.
Dostoiewsky, iris, 383.
Double imbedding in celloidin and paraffin, 112.
Double stains, *see* Stains, Combination.
Doyère, nerve and muscle of Arctiscoida, 475.
Drasch, gold staining, 252; tactile hairs, 373.
Drash, serial section method, 122.
Droost, Mollusca, 471.
Druebin, blood-plates, 461.
Dunham, clearing celloidin sections, 109.
Du Plessis, Nemertians, 479; Protozoa, 499.
Durham, alcohol section-fixing process, 120.
Durig, formaldehyde in Golgi's impregnation, 427.
Duval, silver staining, 246, 248; carmine and anilin blue, 236; collodion imbedding, 100 *et seq.*; embryology of Aves, 341; hardening encephalon, 396; purpurin, 235; paraffin sections, flattening, 91.

E.

Ear, inner, 442, 443.
Eau de Javelle, 322, 330.
Eau de Labarraque, 322, 330.
Ebner, von, decalcification, 325.
Echinodermata, 485—488.
Echinorhyncus, 480.
Echtgelb, 219.
Echtgrün, 403.
Edinger, nerve-centres, 59.
Ehlers, fixing fluid for Annelids, &c., 31.
Ehrenbaum, imbedding method, 115.
Ehrlich, dahlia, 195; hæmatoxylin, 171; methylen blue for nerves, 203; Mastzellen, 445, 447; gentian violet, 192; blood, 456, 459; classification of anilin dyes, 179; of cell granules, 446; neutral red,

The numbers refer to the Pages.

226; acidophilous mixture, 229; mixture for eosinophilous cells, 229; triacid mixture, 223.

Ehrlich-Biondi-Heidenhain stain, 219—223.

Ehrmann, structure of epithelium, 372.

Eichler, labyrinth, 443.

Eijkman, degenerate nerves, 412.

Eisig, fixing mixture, 61; narcotising, 14.

Eisen, serial sections, 128.

Eismond, quieting Infusoria, 495.

Elastic tissue, 450, 451.

Eledone, eyes, 470.

Eliel, gum for labels, 504.

Elschnig, celloidin imbedding, 101.

Embryology, general methods, 331 *et seq.*; Amphibia, 342—344; Amphipoda, 352; Aphides, 351; Araneina, 351; Arthropoda, 348—352; *Astacus*, 352; Aves, 338 *et seq.*; Blattida, 350; Cephalopoda, 346; Diptera, 351; Echinodermata, 487; Gastropoda, 347; Lepidoptera, 350; Mammalia, 335; Mollusca, 346 *et seq.*; Phalangida, 351; Pisces, 344 *et seq.*; Planaria, 352; Reptilia, 342; Teleostea, 344—346; Tunicata, 346; Vermes, 352 *et seq.*

Embryos, reconstruction from sections, 333.

Emery, injection, 309.

Encephalon, 385 *et seq.*; and *see* Central nervous system, neurological methods, &c.

Endosmosis, to avoid, 4.

Engelmann, ciliated epithelium, 471; green light in microscopy, 504.

Entz, G., Infusoria, 498.

Eosin, 226; eosin stains for blood, 458, 459.

Epidermis, 370.

Epithelium, 370 *et seq.*; ciliated, 471; glandular, 370.

Erlicki, hardening fluid, 59.

Errera, nigrosin, 197.

Erythrosin, 226.

Essence, *see* Oil, and Clearing agents.

Eternod, histological rings, 244.

Ether, for preserving, 5.

Etherisation, 14, 354.

Eulenstein, cement, 286.

Eunice, 484.

Everard, Demoor and Massart, hæmatoxylic eosin, 239.

Examination media, 262 *et seq.*

Exner, hardening brain tissue, 391; process for medullated nerve tracts, 412.

Exosmosis, to avoid, 4.

Eycleshrimer, orientation of celloidin objects, 103; cutting same, 110; clearing, 109; serial sections, 129.

Eyes of Arthropoda, 473 *et seq.*; of Cephalopoda and Heteropoda, 470; of Chitonidæ, 470; of Gastropoda, 469; of *Pecten*, 470; of Asteroidea, 486.

F.

Fabre-Domergue, glucose preservative medium, 270; Infusoria, 497, 499.

Fairchild, dehydration cylinders, 4.

Fajerstajn, nerve-endings in tongue of frog, 375.

Faris, glycero-gum, 269.

Farrant's medium, 268.

Fast green, 228; fast blue, 228.

Fat, 35, 445.

Faussek, critique of Flemming's mixture, 32.

Fecundation, artificial, 331.

Feist, methylen-blue method, 210—212; orientating spinal cord, 401.

Ferreri, phloroglucin decalcification, 326, 327.

Ferria, elastic tissue, 451.

Ferrier, examination liquid for blood, 459.

Fick, Golgi's silver chromate method, 429.

Fiedler, *Spongilla*, 494.

Field and Martin, petroleum-ether for imbedding, 80; orientation in paraffin, 85; double imbedding, 113; Schällibaum's collodion, 121.

The numbers refer to the Pages.

FISCHEL, medullated nerve, 404.

FISCHER, soap imbedding mass, 477; Trematodes, 476; tactile corpuscles, 373; motor plates, 379.

FISCHER, A., cell granules, 369.

FISH, oil of thyme, 69, 109; celloidin section-cutting, 110; decalcification, 323, 324, 326; hardening brain, 391, 396, 397, 398; formaldehyde for Golgi's impregnation, 427.

Fixing, generalities, 2 *et seq.*, 18 *et seq.*

Fixing agents, 24 *et seq.*; action of, 18; choice of, 19; methods of using, 20; washing out, 2, 20; cytological, 360—365.

FLATAU, Golgi's sublimate impregnation, 433.

Flattening sections, 87, 91.

Flagella, stains for, 499.

FLECHSIG, gold method, 256; redwood nerve stain, 411; other nerve stains, 434.

FLEMMING, chromo-acetic acid, 30; chromo-aceto-osmic acid, weak solution, 31; strong ditto, 33; cytological methods, 354, 355, 357, 360, 363; damar solution, 281; double stains, 216, 217; eyes of Gastropods, 469; fixing liquids, 30, 31, 33, 50, 361; preservation in glycerin mixtures, 5; injection of molluscs, 471; staining method, 182 *et seq.*, 189 *et seq.*, 195; safranin solution, 190; gentian violet, 192; dahlia, 195; orange method, 217; fatty tissue, 445; bone, 454; goblet cells, 463; connective-tissue fibrils, 444.

FLESCH, MAX, chromo-osmic mixture, 31; dichroism of hæmatoxylin stains, 165; inner ear, 443; modification of Weigert's hæmatoxylin stain, 407; dry preservative process, 439; dental tissue, 456; blood, 457.

FLÖGEL, serial sections, 126.

FLORMAN, celloidin imbedding, 106.

Flustra, 468.

FOÀ, fixing liquid, 42; hæmatoxylin and safranin stain, 240; blood, 460.

FOETTINGER, chloral hydrate narcotisation method, 14; prepared paraffin, 94.

FOL, absolute alcohol, 52; albumen fixative, 124; bleaching osmic objects, 27, 28; chromo-aceto-osmic acid, 32; cleaning slides, 503; decalcification, 326; gelatin fixative, 128; glycerin jelly, 295; gold-impregnation of marine animals, 258; gumming labels, 504; imbedding *in vacuo*, 83; injection masses, 299, 303, 305, 306; Infusoria, 499; narcotisation method, 17; paraffin oven, 82; perchloride of iron fixing and staining process, 44, 260; picro-carmine, 154; picro-chromic acid, 36; reconstructing sections, 333.

Formaldehyde, properties, 53; for fixing, 53; hardening, 62; preserving, 466; macerating, 314; hardening brain, 397, 398; for the Golgi impregnation, 427; as a mordant, 187.

Formalin, *see* Formaldehyde.

Formalose, *see* Formaldehyde.

Formic acid for fixing, 46.

Formol, *see* Formaldehyde.

FOSTER and BALFOUR, embryology of Aves, 338, 340.

Fowl, embryology of, 338, 341.

FRANCOTTE, section-stretcher, 87; vacuum imbedding, 83.

FREEBORN, staining nervous centres, 416; connective tissue, 444.

Freezing microtome, masses for, 115—118.

French cement, 287.

FRENKEL, fixing mixture, 43.

FRENZEL, serial section methods, 126, 128; sublimate solutions, 38.

FREY, injection mass, 305; iodised serum, 264; indifferent liquids, 263.

FRIEDLAENDER, fixing mixture, 493; critique of Golgi's impregnation, 420.

Frog, bladder, 384; tongue, 375.

Fuchsin, basic, 196; ZIEHL's carbolic, 196.

The numbers refer to the Pages.

Fuchsin S, *see* Säurefuchsin, and Acid fuchsin.

G.

Gage, picro-carmine, 154; chloral hæmatoxylin, 173; decalcification, 325; section stretcher, 87; preservative fluid, 266; injection, 310; smooth muscle, 382; section-fixing, 122, 129; clearing mixture, 69; picric alcohol, 50; preservation of potash or soda preparations, 314; macerations, 314, 317.

Galeotti, *intra-vitam* staining, 139; neutral red, 226.

Gallemaerts, section-fixing process, 121.

Galli, stain for neuroceratin funnels, 403.

Gannal, mounting medium, 265.

Garbini, safranin staining, 191; Alcyonaria, 489; cleaning slides, 503.

Gaskell, paraffin sections, water method, 91.

Gastropoda, embryology, 347; eyes, 469; preparation, 468 *et seq.*; killing, 16, 468.

Gaule, section-fixing process, 119; hardening brain, 392; fixing liquid, 40; choice of paraffin, 94.

Gay-Lussac, table for diluting alcohol, 502.

Geberg, gold method, 257; corpuscles of Grandry, 374.

Gedoelst, digestion, 321; medullated nerve, 404.

Gehuchten, van, acetic alcohol, 45; cytological methods, 364; nerve centres, 399, 403.

Gelatin, imbedding masses, 97, 98, 117; injection masses, 293 *et seq.*; cement, 285; section fixative, 127.

Gelpke, Weigert's hæmatoxylin, 406.

Gentian blue, 230.

Gentian violet, 192—194.

Geoffroy, mounting medium, 275.

Geonemertes, 479.

Gephyrea, 481.

Gerlach, gold method, 255, 256; gelatin imbedding, 98; injection, 300; embryology of Aves, 339; nerve-endings in muscle, 379.

Géza Entz, Infusoria, 498.

Giacomini, gelatin process for serial sections, 134; dry process for preserving brains, 437.

Gibbes, Heneage, borax-carmine, 155; cleaning slides, 503.

Gierke, maceration of nerve tissue, 316; staining nervous centres, 416; action of chloral on ditto, 58; uranium carmine, 155; impregnation, 242.

Giesbrecht, clearing method, 64; serial section method, 122; section-stretcher, 87; paraffin imbedding, 81, 82.

Gieson, van, clearing celloidin sections, 109; formaldehyde, 397.

Gilson, acetate of uranium fixing liquid, 46; fixing with osmic acid, 26; bleaching bichromate objects, 58, 330; celloidin imbedding, 111; chloral hydrate jelly, 275; double imbedding, 113; mercuro-nitric fixing mixture, 40; preservative fluid, 266.

Glandular structures, 461 *et seq.*

Glucose preservative media, 270.

Glue, marine, 287.

Glycerin, 270 *et seq.*; glycerin mounts, to close, 285; injections, 308 *et seq.*; glycerin jelly, 97, 98, 273 *et seq.*; preservative mixture, 272.

Glycero-gum, 269.

Glychæmalum, 169.

Goadby, preservative fluids, 267.

Goblet cells, 463.

Goette, hardening liquid, 344.

Gold chloride, for impregnation, generalities, 250 *et seq.*; composition of the commercial salt, 251; and see the names of Authors.

Gold orange, 219.

Gold size, 287.

The numbers refer to the Pages.

Golgi, chromate of silver impregnation, introduction, 419; slow method, 421—423; rapid method, 423; mixed method, 424; critique of the same, 424 *et seq.;* variations, 425—431; fixation of nerve-centres by injection, 387; mercuric impregnation of nervous centres, 431; corpuscles of Golgi, 381, 382; nerve-endings in muscle, 381.

Goodall, spinal cord, 399.

Gorgonia, 489.

Goronowitsch, embryology of Teleostea, 345.

Graf, fixing Hirudinea, 485.

Graff, von, Turbellaria, 477.

Gram, stain for bacteria, 193.

Grandry, corpuscles of, 374.

Granule cells, 445 *et seq.*

Granules, protoplasmic, 368, 445 *et seq.*, 459, 460.

Grape-sugar imbedding mass, 114.

Graser, staining process, 201.

Gravis, section, fixative, 127.

Gray, serial sections, 127.

Green light in microscopy, 504.

Grenacher, alum-carmine, 150; alcoholic carmine, 157; bleaching mixture, 330; borax-carmine, aqueous, 155; alcoholic, 156; castor oil for mounting, 278; hæmatoxylin, 170; purpurin, 235; eyes of Mollusca, &c., 470.

Greppin, Golgi's silver chromate method, 430.

Grieb, alum-carmine, 150.

Griesbach, benzo-purpurin, 225; Bengal rose, 226; Biebricher Scharlach, 226; blood-stains, 456, 457; Congo red, 225; elastic tissue, 450; iodine green, 228; metanil yellow, 219; methyl green, 200; gold orange, 219.

Grouven, Ehrlich-Biondi mixture, 222.

Grübler, histological reagents, 144; cherry gum, 495.

Grünpulver, 197.

Grünstichblau, 230.

Guernsey blue, 228.

Guignet, soluble Prussian blue, 301.

Gulland, flattening and mounting paraffin sections, 91, 120; blood, 460.

Gum, arabic, pure, 126; preservative media, 268 *et seq.;* imbedding masses, 113, 114, 116, 117; injection mass, 307; mucilage for labels, 504; fixative for sections, 126.

Gum damar, 280.

Gum sandarac, 283.

Gutta-percha, cement, 287; section-fixing process, 128.

H.

Hacke, hæmatoxylin, 173.

Hæmacalcium, 172.

Hæmalum, 167, 168.

Hæmateate of ammonia, 164.

Hæmateïn, generalities, 161—167; properties, 163; where obtainable, 163; formulæ for hæmateïn stains, 167 *et seq.*; alumina-hæmateïn stains, 167—173; other hæmateïn stains, 173—178; and *see* Hæmatoxylin.

Hæmateïn-ammoniak, 163.

Hæmateïnum crystallisatum, 163.

Hæmatoxylic eosin, 238.

Hæmatoxylin, generalities, 161—167; theory of staining with, 161; ripening of solutions, 161—163; dichroism of the stains, 164, 165; affinities for plasma granules and for mucin, 165; the practice of staining, 164—166; how to "blue" stains, 164, 165; formulæ for stains:—alum solutions, 167—173; stains depending on the formation of other lakes, 173—178, 367; combination stains, 237 *et seq.;* and *see* the separate entries, such as "Iron hæmatoxylin," "Hæmacalcium," &c.; and *see* Hæmateïn.

Hæmoglobin, 458.

Haensel, liquid of, 36.

Hairs, tactile and others, 372.

The numbers refer to the Pages.

Halicryptus, 481.

Halle and **Born**, orienting celloidin objects, 103.

Haller, Béla, macerating fluid, 318.

Halliburton, chemistry of the cell, 359.

Hamann, carmine, 155; Echinoderms, 486; Acanthocephali, 480.

Hamilton, freezing process, 116; hardening brain, 395; freezing ditto, 399.

Hanaman, cleaning slides, 503.

Hansen, hæmatein stain, 169; elastic tissue, 451.

Häntsch, glycerin medium, 273.

Hardening, generalities, 21; practice of, 22; nerve-centres, 388, 398.

Hardening agents, 56 *et seq.;* choice of, 23.

Hardy, Rotifers, 482.

Harmer, silver-staining marine animals, 249.

Hartig, white injection mass, 305.

Harting, preservative fluid, 266; cement, 287.

Haswell's dehydration apparatus, 4.

Haug, borax-carmine, 155; hæmatoxylin, 327; decalcification, 323, 325, 326; modification of Weigert's nerve-stain, 412.

Hayem, blood, 456, 457.

Heat, killing by, 11; fixing by, 365.

Heidenhain, M., cytological methods, 355, 364, 366; lanthanin, 364; hæmatoxylin (iron), 175, 366; discussion as to the Ehrlich-Biondi stain, 220, 222; paraffin imbedding, 80; cause of "shrinkage" in paraffin sections, 80, 87; sublimate solution, 40; water section-fixing process, 120; thionin, 194; Bordeaux-iron-hæmatoxylin, 366; vanadium hæmatoxylin, 368.

Heidenhain, R., hæmatoxylin, 173; glands of small intestine, 465.

Heider, collodionising sections, 90.

Helix, eyes, 469; ova, 347; narcotisation, 15, 16.

Heller and **Gumpertz**, osmic acid impregnation, 260.

Henchman, embryology of *Limax*, 347.

Henking, collodionising sections, 90; embryology of Diptera, 351; of Phalangida, 351; of Insecta in general, 348; examination liquid for ova, &c., 349.

Henle, staining nervous tissue, 415.

Henneguy, alum-carmine, 151; embryology of Aves, 340; of *Axolotl*, 343; of Gastropoda, 347; of Teleostea, 344; Protozoa, 495, 496; washing out carmine stains, 148; permanganate staining method, 186; mounting methyl green stains, 199; mounting paraffin sections, 91, 125, 127; L. Johnson's platinic mixture, 59, 60; staining with thionin, 195; restaining old mounts, 505.

Hénocque, gold process, 255.

Herbst, corpuscles of, 374.

Herla, stain for attraction spheres, 368.

Hermann, silver staining, 246; papillæ foliatæ of rabbit, 375; platino-aceto-osmic mixture, 43, 362; osmium and pyroligneous acid impregnation, 258; cytological stains, 367; formaldehyde, 53, 54, 62, 63.

Hermann-Böttcher staining process, 182, note.

Herrick, cochineal alum-carmine, 151.

Hertwig, silver staining, 246, 249; macerating methods, 318; Actiniæ, 488; Medusæ, 491; embryology of *Rana*, 343; of *Triton*, 343; tobacco-smoke method, 12.

Herxheimer, elastic tissue, 450; epithelium, plasma fibrils of, 372.

Heschl, amyloid matter, 199.

Hessert, stains for flagella, 500.

Heteropoda, eyes, 470.

Heurck, van, mounting medium, 277.

Heydenreich, amber varnish, 289.

Heymons, embryology of Blattida, 351.

Heys, balsam, 279.

The numbers refer to the Pages.

Hickson, hæmatoxylin and eosin, 237; maceration, 314; eye of *Musca*, 473.
Hirota, blastoderm of fowl, 341.
Hirudinea, 15, 208, 484.
His, fixing, 35; cornea, 376.
Hochstetter, injection, 310.
Hofer, narcotisation method, 15.
Hoffmann, vacuum imbedding, 83.
Hofmann, ovum of fowl, 340.
"Hofmann's Grün," 228.
Hoggan, silver staining, 246; histological rings, 244; perchloride of iron stain, 260.
Holl, toluol for imbedding, 79; note concerning liquid of Kleinenberg, 361.
Holothurioidea, 485.
Homarus, eyes, 474.
Hopewell Smith, decalcification, 325; teeth, 453.
Hopkins, maceration, 318.
Horn, 372.
Hoyer, picro-carmine, 154; silver staining, 246; gold ditto, 255; gum-arabic mounting medium, 268; gelatin injection masses—carmine, 298; Berlin blue, 303; lead chromate, 304; silver nitrate, 305; green, 305; shellac mass, 310; oil-colour masses, 310; chloral for preserving masses, 294; water section-fixing process, 120; reactions of mucin, 461.
Hoyer, jun., formaldehyde, 54.
Huber, Golgi's silver chromate method, 429; medullated nerve, 404.
Hudson, Rotifers, 482.
Hyatt, imbedding method, 114.
Hydatina, 482.
Hydra, 15, 490.
Hydrate of chloral for narcotising, 14; and *see* Chloral.
Hydrochlorate of cocain, 15.
Hydrochloric acid for decalcification, 323, 325.
Hydrofluoric acid for desilicification, 327.
Hydrogen peroxide for bleaching, 25, 28, 30, 329.
Hydroidea, 490.
Hydroxylamin, 15.
Hypochlorite of potassium, *see* Eau de Javelle.
Hypochlorite of sodium, *see* Eau de Labarraque.

I.

Ide, double imbedding, 113; epithelium, 371.
Igacuschi, gold process for liver, 464.
Iijima, embryology of *Planaria*, 352.
Imbedding defined, 74; generalities on, 74 *et seq.*; double imbedding in celloidin and paraffin, 112; and *see* also Celloidin, Paraffin, &c.
Impregnation, distinguished from staining, 241; negative and positive ditto, 241; primary and secondary ditto, 242.
Impregnation methods, 241 *et seq.*; and *see* Gold chloride, Nitrate of silver, Methylen blue, &c.
Inchiostro di Leonardi, 200.
India-rubber cement, 287.
Indian ink injection, 310.
Indifferent liquids, 262 *et seq.*
Indigen, 228.
Indigo substitute, 228.
Indigo-carmine, 235; **Thiersch**'s staining method, 235; **Seiler**'s, 235; **Merkel**'s, 236; with safranin, 229.
Indirect staining method, 137 *et seq.*
Indulin, 228.
Indulin-aurantia-eosin mixture, 229.
Infusoria, 495 *et seq.*, and *see* Protozoa.
Injection of Mollusca, 471.
Injections, 293 *et seq.*; injection masses,—*albuminous*, 307; *aqueous*, 309 *et seq.*; *collodion*, 310; *fatty*, 310; *gelatinous*, 293 *et seq.*; *glycerin*, 308 *et seq.*; *gum*, 307; *resinous*, 310; *starch*, 310; and *see* the names of Authors.
Injections, natural, 311.
Inner ear, 442, 443.
Insecta, *see* Arthropoda.
Insecta, embryology, 348 *et seq.*

The numbers refer to the Pages.

Intra-epidermic nerve-fibres, 373.

Intra-vitam staining, 138, 203.

Inversion of stains, 142, 224.

Iodic acid for fixation, 458.

Iodide of palladium nerve stain, 413.

Iodine for fixing, 46, 47; for hardening, 62; LUGOL'S solution, 46; iodine as a mordant, 141, 143.

Iodine green, 228.

Iodine hæmatoxylin, 173.

Iodised serum, 263, 264; ditto for macerations, 313.

Iris, 383.

Iron, perchloride, for fixing, 44; for staining, 260; pyrogallate of, 260; iron-hæmatoxylin stains, 175—177; with Bordeaux, 366; iron-carmine, 152; detection of iron in cells, 359.

ISRAEL, orceïn, 234.

J.

JACKSON, cloudiness in clearing media, 65.

JACOBS, freezing mass, 117.

JACQUET, injection of leeches, 485.

JADASSOHN, structure of epithelium, 272; thionin for plasma-cells, 450.

JÄGER, glycerin medium, 273.

JAKIMOVITCH, silver staining, 248; medullated nerves, 404.

JAMES, cleaning slides, 504; damar solutions, 281.

JANSSENS, carmine blue, 231.

JAVELLE, eau de, *see* Eau de Javelle.

JELGERSMA, staining nervous centres, 416.

JELINEK, extraction of picric acid, 47; Stabilit imbedding blocks, 108.

Jellies, glycerin, 273—275.

JENSEN, immobilisation of Infusoria, 496.

JOHNSON, LINDSAY, keeping osmic solutions, 24; sunning solutions of metallic salts, 24, 243; platinic hardening mixture, 59; gold impregnation, 257; retina, 439, 440; cementing celloidin blocks, 107.

JOLIET, imbedding method, 113.

JOSEPH, injection mass, 307.

JULIEN, cement, 289; stains for flagella, 500.

JUNG, microtomes, 71.

K.

KADYI, imbedding mass, 95.

KAES, nerve stain, 411.

KAISER, glycerin jelly, 97, 274; naphthylamin brown for spinal cord, 417; Bismarck-brown stain, 196; nerve stain, 410.

KALLIUS, Golgi's silver chromate method, 426, 431.

KANTHACK, histological reagents and apparatus, 144.

KASTSCHENKO, reconstruction from sections, 333.

KEISER, sublimate solution, 40; fixation of Acanthocephali, 481.

KEMP, blood-plates, 460.

KENT, S., Infusoria, 46, 497.

KENT, A. F. S., cement, 290.

Kernschwarz, 233.

Killing tissues and organisms, 11—17.

KINGSLEY, imbedding method, 77; section-fixing, 123.

KIONKA, blastoderm of fowl, 341.

KISHINOUYE, embryology of Araneina, 351.

KITTON, asphalt, 286; cement, 290.

KLEBS, glycerin jelly, 97.

KLEIN, fixing mixture, 30; cornea, 375; cochineal, 151.

KLEINENBERG, colophonium for mounting, 281; embryology of *Lumbricus*, 352; picro-sulphuric acid, 48; hæmatoxylin, 173.

KLEMENSIEWICS, picro-carmine, 154.

KNAUER, cleaning slides, 504.

Knotting, for cement, 287.

KOCH, VON, copal imbedding method, 114.

KOFOID, embryology of *Agriolimax*, 348.

KOGANEI, iris, 383.

Kölliker, ovum of rabbit, 335, 337; bone, 454.

Kollmann, fixing mixture, 345.

Kolossow, gold method, 256; osmic acid mixture, 27; osmic acid stain, 260; regeneration of osmic acid solutions, 25.

Köppen, elastic tissue, 451.

Kopsch, formaldehyde for Golgi's impregnation, 427.

Korotneff, killing Siphonophora, 13.

Korschelt, Infusoria, 497.

Korybutt-Daszkiewicz, plasma-cells, 446.

Kossel, chemistry of the cell, 359.

Kossinski, double stains, 229, 230.

Kotlarewsky, central nervous system, 32, 61, 392.

Kowalewsky, embryology of Teleostea, 345.

Krause, corpuscles of, 375; molybdate of ammonium, 261; motor plates, 381; silver staining, 248; retina, 441, 442; thiophen green, 228; Ehrlich-Biondi mixture, 219.

Kreasote, for bleaching, 329; for clearing, 70, 109.

Kromayer, plasma fibrils of epithelium, 371.

Kronecker. artificial serum, 265.

Krönig, cement, 288.

Krysinsky, photoxylin imbedding, 100.

Kühne, digestion fluids, 321; macerating mixture, 318; motor plates, 381.

Kühne, H., aniseed freezing mass, 118.

Kuhnt, retina, 441.

Kükenthal, Annelids, 14, 483, 484.

Kultschitzky, fixing mixture, 36; double imbedding, 112; preservative fluids, 5; tactile corpuscles, 374; stain for nervous centres with hæmatoxylin, 410; neuroglia, 436; elastic tissue, 451; "patentsaures Rubin," 219.

Kupffer, embryological methods, 342; axis-cylinder, 403; liver, 464.

Kuskow, digestion fluid, 320.

L.

Labarraque, eau de, *see* Eau de Labarraque.

Labelling slides, 504.

Labyrinth of ear, 442, 443.

Lacerta, ova of, 342.

Lachi, formaldehyde for brain, 397.

Lactic acid for decalcification, 324, 326.

Lamansky, sensibility of the eye, 505.

Lamellibranchiata, 468, 471.

Landois, impregnation methods, 261; macerating mixture, 316.

Landolt, retina, 442.

Landsberg, Infusoria, 497.

Lang, fixing liquids, 40; methods for Platyhelmia, 40.

Langerhans, *Amphioxus*, 378; gum and glycerin medium, 269; tactile corpuscles, 374; fixation of marine animals, 21.

Langhans, dichroism of hæmatoxylin stains, 165.

Lankester, eyes of *Limulus*, 473.

Lanthanin, 364.

Lauth's violet, 194.

Lavdowsky, blood, 458; sandarac medium, 283; chloral hydrate medium, 265; macerating fluid, 314; inner ear, 442; formaldehyde, 55.

Lawrence, glycerin jelly, 273.

Lead, acetate, 61, 392; chromate, 261.

Leber, impregnation methods, 261; retina, 441.

Lecithin, reactions, 357.

Leclercq, stains for blood, 459.

Lee, A. B., preservation in paraffin or cedar oil, 5; cedar oil for paraffin imbedding, 80; mounting celloidin sections, 128; *intra-vitam* staining, 139, 140; sublimate solutions, 38, 364; formaldehyde, 53, 54; celloidin imbedding, 101, 105, 106; dry celloidin process, 111; Mayer's albumen, 125, 128; toluidin blue, 197; malachite green, 228; nigrosin, 228; bleu de Lyon, 230; Wasserblau, 231; Kernschwarz, 233; orceïn, 235; carmine and indigo-carmine, 235; impregnation with gold, 250; osmic acid and pyrogallol stain, 258; glycerin mixture, 272; resinous mounting media, 277—280; colophonium mounting medium, 282; cedar oil for mounting, 278; paper cells for mounting, 285; Nemerteans, preparation, 478; fixing Hirudinea, 485.

Leech, 485.

LEGAL, alum-carmine, 152.

LEGROS, silver staining, 248.

Lemon juice for fixing nuclei, 364; for killing, 12.

LENDENFELD, VON, staining collar-cells, 494.

LENHOSSÉK, VON, tongue of rabbit, 375; nerves of annelids, 484.

LENNOX, retina, 441.

Lens, crystalline, 315, 377.

LEONARDI, inchiostro di, 200.

Lepidoptera, embryology, 350.

LEPKOWSKY, gold method for teeth and bone, 454.

LETELLIER, injection, 310.

LEUCKHART, imbedding boxes, 76.

LEVULOSE for mounting, 270.

LEWIS, BEVAN, freezing process for brain, 388; hardening ditto, 394; staining, 416.

Lichtgrün, 227.

LIEBERMANN, analysis of carmine, 145; carminic acid, 146.

Light, action of, on alcohol containing chromic objects, 30; on metallic salts, 242; green, in microscopy, 504.

Ligula, 476.

LILIENFELD, blood-plates, 461; chemistry of the cell, 359.

LILIENFELD and MONTI, test for phosphorus in tissues, 359.

Limax, 469; embryology, 347.

Limulus, 473.

LINDSAY JOHNSON, *see* JOHNSON, LINDSAY.

Linseed-oil injection, 310.

Liquid of Müller, of Erlicki, of Kultschizky, of Merkel, &c., *see* the names of the respective Authors.

Liquidambar, 283.

LIST, hæmatoxylin and eosin, 237; methyl green and eosin, 226; other combination stains, 464; macerations, 313; Anthozoa, 488; Coccidæ, 472; goblet cells, 464.

Lithium-carmine, 155.

Litmus paper, to make, 297.

Liver, 464.

LO BIANCO, S., *see* BIANCO, S. LO.

LOCY, embryology of *Agelena*, 351.

LOEFFLER, stains for flagella, 499.

Loligo, eyes, 470.

LONGHI, eserin for Infusoria, 499.

LONGWORTH, corpuscles of Krause, 375.

LÖNNBERG, cytology of the snail, 356; *Triænophorus*, 476

LOOSS, softening chitin, 322; Nematodes, 479.

LOVETT, cement, 290.

LÖWE, crystalline, 377.

LÖWENTHAL, nerve-centres, 59; picrocarmine, 154.

LÖWIT, gold method, 252; blood, 456, 457, 459.

LÖWY, macerating skin, 371.

Loxosoma, 468.

LUGOL, solution of, 46.

Lumbricus, 483; embryology, 352.

LUSTGARTEN, elastic tissue, 195, 450.

LUYS, noir Colin, 416.

Lymphatics, *see* Impregnation methods and Injections.

Lysol, properties, 319; for maceration, 319.

M.

MAAS, carmine and malachite green, 237.

MACALLUM, *Sphyranura*, 477; double stain, 236; detection of iron in cells, 359.

MACBRIDE, development of *Amphiura*, 488.

Maceration, 312 *et seq.*; epithelium, 370; Mollusca, 471.

Macrotoma, 475.

Magdala red, 195.

Magenta, 196; Magenta S, 219.

MAGINI, stain for nervous centres, 434.

The numbers refer to the Pages.

Magnesium chloride, for narcotisation, 16; sulphate, *ibid.*

MÄHRENTHAL, VON, osmium impregnation, 260.

Malachite green, 228.

MALASSEZ, ammonia-carmine, 155.

MALFATTI, chemistry of the cell, 359.

MALLORY, phospho-molybdic acid hæmatoxylin, 178, 418.

Mammalia, embryology, 335 *et seq.*

Manchester brown, 200.

MANFREDI, gold method, 256.

Manganese chloride, 263.

MANN, osmic acid mixture, 27; picro-sublimate mixtures, 41; toluidin blue, 197; Wasserblau and eosin, 231; fixative for sections, 126; fixing nerve-centres, 387.

MARCHI, VON, *Limax*, 469; corpuscles of Golgi, 381; degenerate nerves, 412.

MARCUS, formaldehyde for nerve-centres, 397.

Marine animals, killing, 17; fixing, 21; silver impregnation, 248; gold ditto, 258.

Marine glue, 287.

MARK, collodionising sections, 89; embryology of *Limax*, 347; section-fixing, 123.

MARSCHALKÒ, VON, plasma-cells, 450.

MARSH, bleaching, 328; gelatin cement, 285; decalcification mixture, 326.

MARSHALL, Mays' gold process, 381.

MARTIN, benzoazurin, 197, 232.

MARTINOTTI, safranin staining, 191; damar solutions, 281; anilin black, 416; picro-nigrosin, 417; elastic tissue, 450; *intra-vitam* staining, 138.

MASON, nervous system of reptiles, &c., 398.

Mastzellen, 445—450.

MATSCHINSKY, bone, 453.

MAURICE and SCHULGIN, double stain, 237; embryology of *Amarœcium*, 346.

Mauvein, 196.

MAX FLESCH, *see* FLESCH.

MAYER, PAUL, acidulated alcohol, 52; alcoholic carmine, 157, 158; albumen fixative for sections, 123; bleaching, 328; borax-carmine, 156; carmine staining, theory of, 145—147; Carmalum, 149; chloride of aluminium carmine, 150; Paracarmine, 156; carmine and indigo-carmine, 236; cochineal staining, theory of, 147; cochineal, old formula, 158; new formula, 159; desilicification, 327; general principles of preparation, 9; hæmatoxylin (hæmatein) staining, theory of, 161—167; Hæmalum, 167, 168; Hæmacalcium, 172; Glychæmalum, 169; preparation of the ammonia-salt of hæmatein, 164; bluing hæmatein stains, 164, 165; mounting hæmatein stains, 278; critique of Kleinenberg's hæmatoxylin, 173; injection, 309; picro-carmine, 154; picro-sulphuric acid, 48; picro-hydrochloric acid, 50; picro-nitric acid, 50; section-stretcher, 87; section-fixing methods, 123—125; shellac fixative, 123; theory of staining, 137; staining mucus, 462; Mucicarmine, 463; Muchæmatein, 463; water-bath for paraffin, 82.

MAYER, S., "violet B." for connective tissue, 231, 444; methylen-blue method, 207, 209, 212.

MAYS, nerve-endings (gold process), 381.

MAYSEL, Bismarck brown, 200.

Medullated nerve, 403, 404; and *see* Neurological methods.

Medusæ, 490—492.

MEISSNER, corpuscles of, 375.

MERCIER, hæmatoxylin nerve stain, 412; Upson's nerve-impregnations, 436, 437; Golgi's impregnation methods, 419, note.

Mercury bichloride, *see* Corrosive sublimate; biniodide, mounting medium, 275; cyanide, for fixing, 481; nitrate and picrate, 364.

The numbers refer to the Pages.

Merk, making up chromo-aceto-osmic acid, 34.

Merkel, chromo-platinic liquid, 36; impregnation method, 261; carmine and indigo stain, 236; celloidin imbedding, 100; staining nervous tissue, 415.

Metagelatin, 306.

Metallic salts, action of light on solutions, 242.

Metallic stains, 241 *et seq.*

Metanil yellow, 219.

Metcalf, embryology of *Chiton*, 348.

Methanilin green, 197.

Methanilin violet, 200.

Methyl alcohol for narcotising, 14; for safranin staining, 191.

Methyl blue, 230.

Methyl green, 197—200; combination stains with, 226.

Methyl mixture for maceration, 319.

Methyl violet, 200.

Methylal, for dehydration, 5, 211.

Methylated spirit, 501.

Methylen blue, for impregnation, 202—214, 443; generalities, 202; staining *in toto* during life, 203; staining nerve-tissue during life, 204; influence of oxygen and of ammonia, 205; staining by injection or by immersion, 206; the solutions employed, 206; preservation of the stain, 209; impregnation of epithelia, lymph-spaces, &c., 212, 213; of central nervous system, 443; polychromatic, 202; with eosin, 227; borax-methylen blue, 365; stains for hardened nervous tissue, *see* Rehm, Sahli, and Adamkiewics; for plasma-cells, 448.

Meyer, salicylic solutions, 267; silver staining, 249.

Meyer, Semi, methylen blue for central nervous system, 443.

Mibelli, elastic tissue, 451.

Michrochemistry of the cell, 357—360.

Microtomes, 71—74.

Migula, glycerised serum, 265.

Miller, injection, 305; caoutchouc cement, 286.

Milnesium, 475.

Minot, hæmatoxylin stains, 178; clearing celloidin sections, 109; macerating skin, 371; carmine, 155; microtome, 73.

Mitrophanow, gold process, 371; maceration, 371; sense organs of Amphibia, 375; Wasserblau, 231; prickle-cells, 371.

Mitsukuri, embryology of Reptilia, 342.

Möbius, macerating media, 317.

Moerner, tracheal cartilage, 456.

Moleschott, potash or soda solution, 314; Moleschott and Piso Borme's macerating fluid, 314.

Molgula, 467.

Mollusca, 468—471; embryology, 346 *et seq.*

Molluscoida, 11, 18, 19, 468; and *see* Bryozoa.

Molybdate of ammonium, 364.

Monobromide of naphthalin, 277.

Monti, sulphate of copper impregnation method, 434; test for phosphorus in tissues, 359.

Moore, stain for blood, 458.

Moore, V. A., aniseed imbedding, 118.

Mordants for staining, 140—143.

Morgan, ova of Arthropods, 348, 472; removing albumen from ova, 343, 348; test-cells of Ascidians, 346.

Morphia for narcotising, 16.

Moseley, shell, 470; Chitonidæ, 470.

Mosso, blood, 457.

Motor plates, 378 *et seq.*

Mounds of Doyère, 475.

Mounting media, *see* Examination media.

Muchæmatein, 463.

Mucicarmine, 463.

Mucilage for labels, 504.

Mucin, 461 *et seq.*

Mucus, removing, from Mollusca, 469.

Mucus glands, 461 *et seq.*

Müller, blood, 456; hardening liquid, 58; silver staining, 247; injection mass, 309.

The numbers refer to the Pages.

Münder, G., histological reagents, 144.
Munson, chloral hydrate fluid, 266.
Musca eyes, 473.
Muscle, dissociation of, 378; nerve-endings in, 379 *et seq.;* smooth, 382 *et seq.;* sections, 378.
Myelin, 403; myelin stains, 405—414.
Myelon, *see* Central nervous system.

N.

Nails, 372.
Nansen, maceration of nerve-tissue, 316.
Naphtha, for clearing, 70; for imbedding, 79.
Naphthalin, monobromide, 277.
Naphthylamin brown, 417.
Narcotisation, 12—17.
Nathusius, von, hairs and nails, 373.
Nealey, bone, 453.
Nebenkern, *see* Cytological methods, Plasma stains.
Neelsen and **Schiefferdecker**, clearing agents, 65.
Negro, motor plates, 381.
Nematodes, 479.
Nemertina, 478.
Nereis, embryology, 353.
Nerves, degenerate, 412.
Nerves, *see* Neurological methods.
Nerve-endings in muscle, 378 *et seq.;* in skin and others, 373 *et seq.;* methylen-blue method, 204 *et seq.;* and *see* Neurological methods.
Nervous system, central, *see* Central nervous system and Neurological methods.
Nessler, test for ammonia, 298, note.
Nesteroffsky, gold process, 256.
Neuroceratin, 403, 404.
Neuroglia, 435, 436.
Neurological methods, 385—443; introduction, 385, 386; section methods, 387—401; cytological methods of neurology, 401—404; nerve-fibre stains, **Weigert** and others, 405—414; axis-cylinder and protoplasm stains, 415—419; axis-cylinder and protoplasm impregnations (**Golgi** and others), 419—434; and *see* Impregnation methods, Retina, Inner Ear, Nerves, Central nervous system, &c., and the names of Authors.
Neutralisation of carmine masses, 296.
Neutral red, 226.
Neutral tar colours, 179.
Neutrophilous cell-elements, 446.
New green, 228.
Neyt, *see* **van Beneden** and **Neyt**.
Nias, cleaning slides, 504.
Nietzki, carminic acid, 146; hæmatein, 161.
Nicolle and **Cantacuzène**, impregnation method, 261.
Nicotin for narcotisation, 13.
Niessing, fixing mixtures, 363.
Nigranilin, 231.
Nigrosin, 197, 228; with safranin, 230.
Nikiforow, borax-carmine, 155; stain for nervous centres, 414; clearing celloidin sections, 108; acidophilous mixture, 229.
Nissen, gentian violet staining process, 193.
Nissl, stains for nerve-cells, 401, 402; for nerves, 414.
Nitrate of silver, for impregnation, 242 *et seq.;* for marine animals, 248; and *see* the names of Authors.
Nitrate of uranium, fixing mixture with osmium, 27.
Nitric acid for fixing, 35; for hardening, 57, 392; for maceration, 318; for decalcification, 324, 325; for bleaching, 329.
Nitric and chromic acid mixtures, 35.
Nitrite of amyl as a vaso-dilatator for injections, 293.
Noir Colin, 231.
Noll, corrosion, 322; sponge spicules, 494; salicylic acid medium, 268.
Nordmann, plasma-cells, 447.
Normal salt solution, 263.
Norris and **Shakespeare**, carmine and indigo stain, 236.
Nuclein, reactions of, 357—360, and *see* Chromatin.
Nucleus, *see* Cytological methods.
Nucleo-albumins, 358.

The numbers refer to the Pages.

O.

OBERSTEINER, central nervous system, 386; hardening, 391; staining, 415.

OBREGIA, Golgi's silver chromate method, 430; serial section method, 133.

Oceania, 491.

Octopus, eyes, 470.

ODENIUS, tactile hairs, 319.

OGATA, hardening brain, 392.

OHLMACHER, reactions of safranin with iodine and picric acid (*pseudo*-carcinoma corpuscles), 192; section-fixing process, 125; formaldehyde staining process, 187.

Oil, of aniseed, imbedding mass, 118; of bergamot, 68; cedar wood, 80; cloves, 9, 67; origanum, 68, 109; sandal-wood, 69; turpentine, 69; and *see* Clearing agents.

Ommatostrephes, eyes, 470.

One-third alcohol, 51, 313.

Onuphis, 484.

Opal blue, 230.

Ophiuridea, 486.

Opisthobranchiata, 468.

Opisthotrema, 476.

OPPEL, liver, 427, 464.

OPPITZ, silver staining, 248.

Orange G, 216, 217.

Orceïn, 234, 445, 451.

Orchella, 234.

Orchestia, embryology, 352.

Orcin, 234.

Orientation in paraffin; in celloidin, 103.

Origanum oil, 68, 109.

Orseille, 234.

ORTH, methyl violet, 200; lithium-carmine, 155.

OSBORN, embryology of *Triton*, 343; brain of Urodela, 400.

Osmic acid, for fixing, 24—29; for hardening, 57, 391; for macerating, 317; regeneration of solution, 25; for impregnation, 258; blackening of preparations with, 27; how to keep solutions, 24; CORI's solution, 24; KOLOSSOW's solution, 25, 27; osmic acid and alcohol, 27; and chromic acid mixture, 31; and chromic and acetic acid mixture, 31—35; platinic mixture, 43; osmic and oxalic stain, 260; osmic and pyrogallic ditto, 258.

Osmicated cells, 28.

Osmium-blackened fat, 35, 445.

Osmium-carmine, 155.

Osmosis, to avoid, 3, 4.

Ostracoda, 472.

Otocyst of *Mysis*, 475.

Ova, *see* Embryology.

Ovens for paraffin, 82.

OVERTON, bleaching osmic objects, 28; fixing with iodine vapours, 47.

OVIATT, injection method, 294.

Ovum, *see* Cytological methods, and Embryological methods.

OWEN, preservative liquid, 267.

Oxalic acid for maceration, 319.

Oxygenated water, 25, 28, 30, 329.

P.

PACINI, preservative liquids, 266.

PAL, hæmatoxylin stain, 409; staining nervous centres, 433.

PALADINO, iodide of palladium nerve stain, 413.

Palladium chloride, for fixing, 43; decalcifying, 323; hardening, 61; staining, 261.

Palladium iodide nerve stain, 413.

Palythoa, 489.

Pancreatin digestion fluids, 320.

PANETH, making Weigert's hæmatoxylin, 407; goblet-cells, 463.

PANSCH, injection mass, 310.

Paper, imbedding trays, to make, 75, 76; paper cells, 285.

Papillæ foliatæ, 375.

Paracarmine, 156.

Paraffin, imbedding processes, 79 *et seq.*; review of, 92; paraffin masses, 93, 94; solvents of, 79, 91; ovens, 82; paraffin for luting mounts, 289.

Paralinin, 359.

Paris green, 198.

The numbers refer to the Pages.

Paris violet, 200.

Parker, turpentine cement, 287.

Parker, G. H., dehydration methods, 5; methylen-blue method, 211, 474; eyes of scorpions, bleaching, 329; eyes of *Homarus*, 474; of *Astacus*, 474.

Parker and Floyd, formaldehyde for brain, 397.

Parma blue, 230.

Partsch, cochineal stain, 150.

"Patentsaures Rubin," 219.

Patten, embryology of Blattida, 350; orientation in paraffin, 84; eyes of Molluscs and Arthropods, 470; maceration of Mollusca, 471.

Paulsen, goblet cells, 463.

Pecten, eyes of, 470.

Pedicellina, 468.

Pelagic ova, 345.

Pennatulidæ, 489.

Pentacrinus, 488.

Pepsin digestion fluids, 320.

Perchloride of iron, for fixing, 44; for impregnation, 260.

Peremeschko, cytological methods, 354.

Perenyi, fixing fluids, 35, 342.

Pergens, picro-carmine, 154.

Periplaneta, ova of, 350, 472.

Perl, soluble carmine, 155.

Permanganate of potash for washing out carmine stains, 148; for macerating, 317; for mordanting, 186; for reducing silver impregnations, 248.

Perophora, 467.

Peroxide of hydrogen, 25, 28, 30, 329.

Peroxide of sodium, 329.

Perrier, narcotising *Lumbricus*, 483.

Perruthenic acid, 464.

Petroleum-ether, 80.

Pfitzner, safranin solution, 190; damar solution, 281; Infusoria, 497.

Phalangida, 475; ova of, 351.

Phallusia, 467.

Phascolosoma, 481.

Phénicienne, la, 200.

Phenylen brown, 200.

Phloroglucin, 326.

Phloxin, 226.

Phoronis, 481.

Phospho-molybdic-acid hæmatoxylin, 178, 418.

Phosphoric acid for decalcification, 326.

Phosphorus in tissues, test for, 359.

Photoxylin imbedding method, 100.

Phyllosoma, 472.

Physalia, 493.

Physiological salt solution, 263.

Physophora, 493.

Pianese, double-stain for connective tissue, 444; methylen blue and eosin, 227.

Picric acid, fixing fluids, 47 *et seq.*; hardening ditto, 61; staining with, 216; decalcification with, 324, 326.

Picric alcohol, 50, 317.

Picro-alum-carmine, 152.

Picro-carminate of ammonia, 153.

Picro-carminate of soda, 154.

Picro-carmine, 153, 154.

Picro-chromic acid, 36.

Picro-hydrochloric acid, 50.

Picro-nigrosin, 417.

Picro-nitric acid, 50.

Picro-osmic acid, 50.

Picro-platinic mixture, 50.

Picro-sublimate mixtures, 41.

Picro-sulphuric acid, 48.

Pictet, examination liquid, 263.

Piersol, embryology of Mammalia, 338.

Pigment spots, artificial, 59.

Pintner, Tæniæ, 476.

Pisenti, alum-carmine, 150.

Plakina, larvæ, 494.

Planaria, 40; embryology, 352.

Plasma-cells, 445—450.

Plasma fibrils, 371.

Plasma stains, 215—232, 365.

Plastic reconstruction of sections, 333.

Plastin, 359.

Platinic mixture, Merkel's, 36, 61; Lindsay Johnson's, 59.

Platino-aceto-osmic mixture, 43.

Platino-osmo-bichromate mixture, 59.

The numbers refer to the Pages.

Platino-sublimate mixture, 332.
Platinum chloride, for fixing, 42, 43.
PLATNER, Kernschwarz, 233; medullated nerve, 403.
Platyhelmia, 476—479.
PLESSIS, DU, *see* DU PLESSIS.
Pluteus, 487.
PODWYSSOZKI, fixing mixture, 34; safranin staining, 191; carcinoma corpuscles, 192.
Poisoning methods, 16.
POLAILLON, impregnation method, 260.
POLI, serial sections, 128.
POLITZER, inner ear, 443.
Polychæta, 484.
Polychromatic methylen blue, 282.
PÖLZAM, imbedding mass, 95.
Porifera, 493.
Porpita, 493.
Potash acetate, mounting medium. 265.
Potash solution for maceration, 314.
Potassium bichromate 58 *et seq.*
Potassium hypochlorite, 322, 330.
Potassium permanganate, *see* Permanganate of potash.
POUCHET, bleaching methods, 329.
PRENANT, safranin staining, 191; cochlea, 442.
Preservative media, 4, 5, 262 *et seq.*
Prickle-cells, 371.
Primrose, 226.
PRINGLE, the water section-fixing process, 120; vacuum imbedding, 83.
PRITCHARD, hardening fluid, 57; reducing solution, 256; inner ear, 442.
Progressive staining, 136, 197 *et seq.*
Prosobranchiata, 468.
Protula, 484.
Protozoa, introductory, 495; quieting, 495; staining *intra vitam*, 496; cilia, 499; flagella, 499; other methods, 497—499.
PRUDDEN, hæmatoxylin, 170.
Prussian blue, soluble, 301; impregnation with, 261; injection masses, *see* Injections.
Prussic acid, for killing, 16.
Pteropoda, 469.
PURCELL, eyes of Phalangida, 475.
Purpurin, 235.
Pyrenin, 359.
Pyridin for hardening and clearing, 62.
Pyrogallate of iron stain, 260.
Pyrosin, 226.

Q.

QUERVAIN, DE, fixation of nerve-centres, 387.
Quieting Infusoria, &c., 495.
Quince-mucilage fixative, 127.
Quinolein blue, 228.

R.

Rabbit, embryology, 335 *et seq.*
RABL, fixing methods, 31, 41, 42, 332; embryos of *Salamandra*, 343; serial sections, 121; alum-cochineal, 151; picro-sublimate mixture, 41; platino-sublimate mixture, 332; medullated nerve, 404; staining method, 239; cytological methods, 363; collodionising sections, 90.
RABL-RÜCKHARD, embryology of Telostea, 345.
RAMÓN Y CAJAL, *see* CAJAL.
Rana, embryology, 343.
RANVIER, alcohol, absolute, preparation of, 52; one-third ditto, 51, 313; areolar tissue, 444; bladder of frog, 384; bone, 451; carmine, neutral, 155; clasmatocytes, 450; cornea, 376; corpuscles of Golgi, 381; decalcification, 325; epithelium, 370; gland-cells, 464; gold chloride impregnation, 253, 254 (and *see* Motor plates, Nerve-fibres, &c.); hæmatoxylin, 173; *injection masses*, carmine, 296; Prussian blue, 300, 301; silver nitrate, 306; glycerin, 309; aqueous, 309; iodised serum, 263; maceration, 313, 319; medullated nerve, 404; motor plates, 380; mucus cells, 464; nerve-fibres, intra-epi-

The numbers refer to the Pages.

dermic, 373; neutral carmine, 155; nitrate of silver impregnation, 243, 245; perruthenic acid, 464; picro-carmine, 153, 154; purpurin, 235; quinoleïn blue, 228; retina, 439, 442; tactile hairs, 373.

Ranvier and Vignal, osmium mixture, 27.

Rawitz, hæmatein staining, 166; "invert" stain, 224; artificial alizarin, 232; Golgi's impregnation memethods, 419, note.

Reagents, how to procure, 143; selected collections of, 503.

Recklinghausen, von, impregnation methods, 242, 246, 247.

Reconstruction of objects from sections, 333.

Redding, gold process, 258.

Redenbaugh, narcotisation, 16.

Regaud, silver impregnation, 247.

Regressive staining, 137, 182 *et seq.*

Rehm, nerve-tissue stains, 402, 417.

Reich, silver nitrate, 246.

Reichenbach, embryology of *Astacus*, 352.

Reinbach, triacid mixture, 223; coverglass preparations, 460.

Reinhold-Giltay microtome, 73.

Reinke, Flemming's orange stain, 218; lysol for maceration, 319; staining horny tissues, 373.

Remak's copper hardening fluid, 344.

Renaut, hæmatoxylin, 173; hæmatoxylic eosin, 238; silver impregnation, 247; cornea, 376.

Reptilia, embryology, 342; myelon, 398.

Resegotti, coal-tar stains, 185, 189; staining by substitution, 185; on the kinds of safranin, and on safranin staining, 189.

Resins, mounting media, 277 *et seq.*; imbedding masses, 114, 115.

Retina, 439—442.

Retterer, natural injections, 311; ova of rabbit, 337; smooth muscle, 382.

Retzius, methylen-blue method, 209.

Rezzonico, medullated nerve, 404.

Rhabdocœla, 477.

Rhizophysa, 493.

Rhopalea, 467.

Ribbon section-cutting, 88.

Richard, Bryozoa, 15.

Rindfleisch, maceration, 317.

Ringing wet mounts, 285.

Ripart and Petit, preservative fluid, 46, 267.

Ritter, embryology of *Chironomus*, 351.

Robert, fixing *Aplysia*, 469.

Robertson, imbedding mass, 114.

Robin, injection masses, 294, 295, 305; natural injections, 311.

Robinski, silver nitrate, 246.

Rocking microtome, 72.

Rollett, Carminroth, 155; freezing process, 118; cornea, 376; sections of muscle, 378.

Roosevelt, pyrogallate of iron, 260.

Rose B. à l'eau, 226.

Rose de naphthaline, 195.

Röse, preparation of bone, 452.

Rosein, 196.

Rosin, stain for nerve-cells, 402; for axis-cylinders and protoplasm, 417.

Rossi, stain for nervous centres, 412; blood, 457.

Rössler, Phalangida, 475.

Rotatoria, 482.

Rouge, fluorescent, 196.

Rouget, silver nitrate, 246, 248; methylen blue, 207.

Rousselet, permanent preservation of Rotifers, 482.

Roux, embryological methods, 344.

Rubin, 196.

Ruffini, corpuscles of Golgi, 382.

Ruprecht, bone, 453.

Ruthenium, impregnation method, 261.

Rutherford, picro-carmine, 154.

Ryder, double imbedding, 112.

S.

Sacerdotti, Golgi's impregnation, 426.

Sachs, motor plates, 381.

Saefftigen, *Echinorhyncus*, 480.

Saffrosin, 226.

The numbers refer to the Pages.

Safranin, 189 *et seq.;* with indigo-carmine or nigrosin, 229, 230; with Wasserblau, 231; with Lichtgrün or Säureviolett, 227; for elastic tissue, 450; for cartilage, 455.

Sagartia, 488.

SAHLI, balsam, 280; nerve-centres, hardening, 390; stain for, 414.

SALA, Golgi's silver chromate impregnation, 430.

Salamandra, embryology, 343; cytology of, 354, 355; breeding larvæ, 355.

Salicylic vinegar, 267, 268.

Saliva, artificial, 315.

Salivary glands, 464.

Salt solution, 263; for maceration, 313, 314.

Salts, metallic, action of light on, 242.

SAMASSA, Golgi's silver chromate method, 429; sections of Ctenophora, 493.

Sandal-wood oil, 69.

Sandarac, gum, 283.

SANFELICE, hæmatoxylin, 173.

SANKEY, staining nervous centres, 416.

SANTORIUS, paraffin stove, 82.

Sapphirina, 472.

SARASIN, embryology of Reptilia, 342.

Sarcolemma, 378.

SARGENT, bleaching, 328.

SATTLER, silver staining, 248.

Säurefuchsin, 219.

Säuregelb, 219.

Säurerubin, 219.

Säureviolett, 227.

SAVILLE KENT, *see* KENT.

SAWTSCHENKO, carcinoma corpuscles, 192.

SAZEPIN, antennæ, 473.

SCHÄFER, muscle-cells, 378.

SCHAFFER, cartilage, 455; retina, 441; reconstructing sections, 333; bone, 451.

SCHÄLLIBAUM, serial sections, 120.

SCHENK, fixing fluid, 46; carbolic fuchsin, 196.

SCHEWIAKOFF, Protozoa, 499.

SCHIEFFERDECKER, cartilage, 456; celloidin imbedding, 100, 101, 104; injection masses, 310; levulose for mounting, 270; methyl mixture, 319; pancreatin digestion fluid, 320; serial sections, 120, 129; maceration of retina, 442; medullated nerve, 404; Mastzellen, 447; the water section-fixing process, 120.

SCHMAUS, uranium-carmine, 155; staining nervous tissue, 416.

SCHMIDT, embryology of *Limax*, 348.

SCHNEIDER, aceto-carmine, 152.

SCHOLTZ, *intra-vitam* staining, 138.

SCHULGIN, double stain, 237; embryology of *Amarœcium*, 346; paraffin mass, 94.

SCHULTZE, F. E., palladium chloride, 613; embryology of Spongiæ, 494; Pennatulidæ, 489; section-stretcher, 87; hardening skin, 370.

SCHULTZE, MAX, acetate of potash mounting medium, 265; sulphuric acid macerating mixture, 319; retina, 442; iodised serum, 263.

SCHÜRMAYER, Infusoria, 495.

SCHWALBE, smooth muscle, 382; cochlea, 442; impregnation, 242.

SCHWARZ, chemistry of the cell, 359.

SCHWARZE, *Cercaria*, 477.

SCHWEIGGER-SEIDEL, carmine, 155.

SCLAVÒ, stains for flagella, 500.

Scorpions, eyes of, 329.

SCOTT and OSBORN, embryology of *Triton*, 343.

Sealing-wax varnish, 290.

SEAMAN, cement, 290; glycerin jelly, 274.

Sections, cutting, 85; chain or ribbon, 88; collodionisation, 89; serial mounting, 119 *et seq.;* section-stretching and section-stretchers, 86—88; unrolling, 87, 91; shrinkage in, 87; reconstruction, 333.

SEELIGER, *Antedon*, 488.

SEGALL, medullated nerve, 404.

SEHRWALD, Golgi's silver chromate method, 428, 429, 431.

SEILER, cleaning slides, 503; alcohol balsam, 280; double stain, 235.

The numbers refer to the Pages.

SELENKA, imbedding method, 77.
Sepia, eyes, 470.
Serial section mounting, 119 *et seq.*
Serum, artificial, 264, 265; iodised, 263, 264, 313.
Shell, 471.
Shellac, cement, 290; imbedding mass, 114; fixative for sections, 122; varnish, 289.
SHIMER, mounting medium, 269.
Shrinkage in elements of sections, 87.
"Siebdosen," 4.
SIEBENMANN, labyrinth, 443.
Sieve-dishes, 4.
SIHLER, nerve-endings, 381.
Silver chromate impregnation, 419 *et seq.*, and *see* GOLGI.
Silver nitrate, *see* Nitrate of silver.
Siphonophora, 492.
Sipunculus, 481.
Skin, hardening, 370; nerves of, 373.
Slides, cleaning, 503; labelling, 504.
SMIRNOW, tactile corpuscles, 374; Golgi impregnation, 426.
SMITH, HOPEWELL, decalcification and sections of teeth, 325, 453.
Soap, imbedding masses, 94 *et seq.*, 477.
SOBETTA, ovum of mouse, 338.
Soda solution for maceration, 314.
Soda hypochlorite, *see* Eau de Labarraque.
Soda-picro-carmine, 154; soda-carmine, 155.
Sodium peroxide, 329.
Solferino, 196.
SOLGER, sarcolemma, 378; salivary glands, 464.
Solid green, 228.
SOLLAS, gelatin imbedding (freezing method), 117.
Soluble blue, 231.
Solution of ERLICKI, of MÜLLER, of MERKEL, &c., *see* the names of the respective Authors; normal salt, 263; macerating salt, 313.
Solutions of metallic salts, how to keep, 242.
SOMMER, *Macrotoma*, 475.
SOULIER, macerating mixtures, 315.
SOUZA, DE, pyridin, 62.
SPEE, GRAF, prepared paraffin, 94.
SPEK, VAN DER, and UNNA, plasma-cells, 449.
Spheres, attraction, *see* Cytological methods, Plasma stains.
Sphyranura, 477.
Spicules of sponges, 494.
Spinal cord, *see* Neurological methods.
Spirit blue, Spirit-soluble blue, 230.
Spirographis, 484.
Spongiæ, 493.
Spongilla, 494.
SQUIRE, histological reagents, 144; oil of origanum, or white thyme, 69; methyl green, 199; commercial salts of gold, 251; glycerin jelly, 275; damar medium, 281; granule cells, 446; Säurefuchsin and orange, 239; mounting methyl-green stains, 199; decalcification, 325, 326; picro-carmine, 154; hæmatoxylin, 171.
Stabilit imbedding-blocks, 108.
STAHL, immobilisation of Infusoria, 496.
Staining, generalities, 135—144; choice of stain, 143; on slide, 7; *in toto*, 7, 10; *intra vitam*, 138, 203, 496; reagents, how to obtain, 143; staining by substitution, 184; substantive and adjective, 140; progressive and regressive, 136; theory of, 137; directions for the regressive method, 182; staining nerve-tissue, *see* Nerves, Central nervous system, Neurological methods, &c.
Stains, combination, 233 *et seq.*, 669.
Stains, general, selective (nuclear, plasmatic, and histologically specific), defined, 135; aqueous and alcoholic stains, 9, 10; effect of transparency and opacity of stains, 188; generalities, 135 *et seq.*; invert stains, 142, 224; cytological, 365; and *see* Anilin black, Anilin blue, Carmine, Cochineal, Eosin, Gold, Hæmatoxylin, Indulin, Iron, Orchella, Purpurin, Safranin, Silver, &c.

The numbers refer to the Pages.

Starch injection mass, 310.

STEINACH, sieve-dishes, 4.

Stentor, 495.

STEPHENSON, mounting medium, 275.

Sternaspidæ, 484.

STIEDA, cements, 290; clearing media, 65.

STILLING and PFITZNER, stomach of *Triton*, 384.

STIRLING, sulphocyanides of ammonium and potasssium, 315; anilin blue-black, 416.

Stomach of *Triton*, 384.

Storax, 283.

Stoves and water-baths, 82.

STRAHL, embryology of Reptilia, 342.

STRASSEN, ZUR, *Bradynema*, 480.

STRASSER, plastic reconstruction, 333; section-stretcher, 87; serial sections, 122.

STRICKER, imbedding mass, 114.

STROEBE, stain for nerve-centres, 414.

STRONG, fixing nerve-centres, 388; formaldehyde for the Golgi impregnation, 427.

Strongylus, 480.

Strychnin for killing, 16.

Styrax, 283.

Sublimate, corrosive, *see* Corrosive sublimate.

Substantive and adjective staining, 140.

Substitution of stains, 184.

SUCHANNEK, the water section-fixing process, 119; bergamot oil, 68; anilin oil for clearing, 70; Venice turpentine, 282; sieve-dishes, 4.

Sulphate of copper fixing fluids, 344, 492, 493; impregnation, 434.

Sulphate of magnesia for narcotisation, 16.

Sulphindigotate of soda or potash, 235.

Sulphocyanides, 315.

Sulphuric acid for maceration, 319.

Sulphurous acid for bleaching, 330.

SUMMERS, serial sections, 122, 129.

"Sunning," solutions of metallic salts, 243.

SUSSDORF, mucus cells, 462.

Sycandra, 494.

Sympodium, 489.

Synapta, 486.

Syrup, imbedding masses, 116; examination media, 265.

T.

Tactile corpuscles, 373 *et seq*.

Tactile hairs, 373.

Tænia, 476; embryology of, 352.

TAFANI, inner ear, 443.

TAGUCHI, injection, 310.

TAL, Golgi's mercury stain, 433.

Tannin solution, 267; for Infusoria, 499.

Tar colours, *see* Anilin dyes.

TARTUFERI, impregnation, corneal cells, 377.

Tegumentary organs of Vertebrates, 370 *et seq*.

TEICHMANN, white injection, 305; oil mass, 310.

Teleostea, embryology of, 344 *et seq*.

Tendon, 381, 382.

Test-cells of Ascidians, 346.

Tethya, 494.

Thenea, 249.

Thermostats, 82.

THIERSCH, carmine injection, 300; Prussian blue ditto, 302; lead chromate ditto, 304; green ditto, 305; oxalic acid indigo-carmine, 235.

Thimbles, paper, to make, 76.

THIN, retina, 442.

Thionin, for plasma-cells, 450; for mucin, 461; as a chromatin stain, 194.

Thiophen green, 228.

THOMA, microtome, 71; decalcification, 325.

THOMPSON'S high refractive medium, 277.

THRELFALL, section-fixing method, 128.

Thyme, oil of, 69.

Tiara, 491.

Tintinnodea, 499.

TIRELLI, medullated nerve, 404.

The numbers refer to the Pages.

Tizzoni, alum-carmine, 150; hardening skin, 370.
Tobacco-smoke killing method, 12.
Toison, blood, 459.
Tolu balsam cement, 290.
Toluidin blue, 197.
Toluol, for clearing, 70; for preserving, 5; for imbedding, 79.
Tornier, hæmatoxylin staining, 174.
Torneux and **Hermann**, silver staining, 246; epithelium, 370.
Trays, paper, to make, 75.
Trematodes, 476.
Trenkmann, stains for flagella, 500.
Triacid mixture, Ehrlich's, 223.
Triænophorus, 476.
Trichinæ, 480.
Trinchese, nerve-endings in muscle, 379.
Triton, stomach, 384.
Tropæolin, 219.
Trypsin, digestion fluid, 321.
Trzebinski, spinal cord, 392.
Tschisch, nerve-centres, 59.
Tubby, celloidin imbedding, 103.
Tullberg, narcotisation with magnesium chloride, 16.
Tunicata, 467; embryology of, 346.
Turbellaria, 477.
Turpentine for clearing, 69; for mounting, 282; cement, 287; for imbedding, 79.

U.

Ulianin, embryology of Amphipoda, 352.
Underwood, gold process, 257.
Unna, bleaching chromic objects, 30; ripening of hæmatoxylin solutions, 161, 163; instanteously ripe hæmatoxylin stain, 163; half-ripe stock solution, 163; plasma-cells and Mastzellen, 448, 449; elastic tissue, 451; mucin, 463; collagen, 444; plasma fibrils of epithelium, 372; stain for smooth muscle, 383.
Upson, gold and iron method, 436; gold and vanadium method, 437; other stains, 416.
Uranium acetate, 27, 46.
Uranium nitrate, 27.
Uranium-carmine, **Gierke** and **Schmaus**, 155.
Ussow, embryology of Cephalopoda, 346.

V.

Vacuum imbedding, 82.
van Beneden, acetic acid fixing method, 44; acetic alcohol, 45; Ascidians, 467; corrosive sublimate liquid, 38; cytological methods, 364; embryology of Mammalia, 335, 337; of Tænia, 352.
van Beneden, and **Neyt**, cytological methods, 45, 228, 364, 368.
van der Spek, Mastzellen, 449.
van der Stricht, bergamot oil, 68.
van Ermengem, stains for flagella, 500.
van Gehuchten, acetic alcohol, 45; cutting brain sections, 399; Nissl's stain, 402; medullated nerve, 403; Golgi's impregnation, 428.
van Gieson, clearing with anilin oil, 109; formaldehyde for brain, 397.
van Heurck, mounting medium, 277.
van Walsem, hot or cool microtome-knife, 88; flattening sections, 91; paraffin mass, 94; serial sections, 128; Weigert nerve-stain, 412.
Vanadate of ammonium, injection mass, 310.
Vanadium hæmatoxylin, 368.
Varnishes, 284 *et seq.*
Velella, 493.
Venice turpentine, for mounting, 282; cement, 287.
Veretillum, 12.
Véridine, 198.
Vermes, 476; embryology, 352 *et seq.*
Vert d'alcali, 198.
Vert d'Eusèbe, 198.
Vert en cristaux, 197.
Vert lumière, 197.
Verworn, narcotisation, 14.
Vesuvin, 200.

The numbers refer to the Pages.

VIALLANES, gold method, 254; celloidin imbedding, 104; brain of Arthropods, 475.
VIALLETON, embryology of Aves, 341.
Victoria blue, 195.
Victoria green, 228.
VIGNAL and RANVIER, osmium mixture, 27; picro-carmine, 153.
VILLE, carmine injection, 296.
Violet B, 231, 444.
Violet of Lauth, 194.
VIRCHOW, action of light on chromic objects, 30.
VIVANTE, bone, 453, 454.
VOGT and YUNG, *Cucumaria*, 486; Rotifers, 482; *Sipunculus*, 481; cleansing intestine of Lumbricus, 483; Cestodes, 476.
VOM RATH, picro-sublimate mixture, 41; picro-sublimate osmic, 42; picro-osmic, 50; picro-platinic, 50.
VON EBNER, decalcification mixture, 325.
VON GRAFF, Turbellaria, 477.
VON KOCH, copal imbedding process, 114.
VON LENDENFELD, staining collar-cells, 494.
VON LENHOSSÉK, nerves of Annelids, 484; tongue of rabbit, 375.
VON MÄHRENTHAL, osmium impregnation, 260.
VON MARCHI, nerve stain, 412.
VON MARSCHALKÒ, plasma cells, 450.
VON NATHUSIUS, hairs and nails, 373.
VON WISTINGHAUSEN, neutralising hæmatoxylin-stained tissues, 166; hæmatoxylin stain, 173; embryology of *Nereis*, 353.
VOSMAER, epithelium of sponges, 249.
VOSSELER, Mayer's albumen fixative, 125; Venice turpentine, 282; wax feet, 312; cardboard ridges for slides, 504.

W.

WADDINGTON, arabin, 126; Infusoria, 499.
Wagnerella, 328.
WALDEYER, inner ear, 442; plasma-cells, 445; decalcification, 323.
WARBURG, Ehrlich-Biondi mixture, 222.
WARD, narcotisation methods, 17; *Sipunculus*, 481.
WASHBURN, embryology of *Limax*, 348.
Washing out, 2, 20, 21.
Wasserblau, 231, 445.
WATASÉ, embryology of Cephalopoda, 347.
Watch-glass imbedding method, 77.
Water, fresh or warm, for killing, 17.
Water section-fixing process, 119.
Water-baths, 82.
Water-blue, 231, 445.
WATNEY, dichroism of hæmatoxylin stains, 165.
Wax feet, 312.
Wax imbedding masses, 94.
WEBB, dextrin freezing mass, 117.
WEBER, preservation of Holothurids, 486; of Asteroidea, 486; of Echinoidea, 486; of Siphonophora, 493; Rotifers, 482.
WEBSTER, naphtha for paraffin imbedding, 79.
WEDL, orchella stain, 234.
WEIGERT, Bismarck brown, 200; picro-carmine, 154; hæmatoxylin nerve methods, 405—412 (the 1885 method, 405; the 1891 method, 407; variations, 409—412); clearing celloidin sections, 109; mounting serial sections, 131; varnish for mounting, without cover, 283; hardening nerve-centres, 390, 397; specific neuroglia stain, 435; fibrin stain, 460.
WEIL, section method, 115; bone, 452.
WEYSSE, embryology of *Sus*, 338.
WHEELER, embryology of Blattida, 350.
WHITE, sections of bone or dental tissue, 453.
Whitelead cement, 290.
White of egg, section-fixing process, 123; imbedding methods, 118; injection mass, 307.

The numbers refer to the Pages.

White zinc cement, 290.
Whitman, fixing mixture, 61; ova of Amphibia, 342; pelagic ova, 345; Hirudinea, 484.
Wickersheimer, preservative fluid, 267.
Will, Aphides, 351.
Wilson, Alcyonaria, 489; embryology of Lumbricus, 352.
Wintersteiner, serial section method, 133.
Wissowsky, blood, 458.
Witt, shellac, 290.
Wlassax, serial section mounting, 120.
Wolff, motor plates, 381; bladder of frog, 384.
Wolters, hæmatoxylin nerve stains, 411, 418; cartilage, 456; skin nerves, 373; vanadium nerve stain, 418.
Woodward, borax-carmine, 155.
Woodworth, orienting paraffin objects, 85.
Wright, *Sphyranura*, 477.

X.

Xylol, for clearing paraffin sections, 70; ditto celloidin sections, 109; for paraffin imbedding, 80; for preserving tissues, 5.

Z.

Zachariadès, bone, 455.
Zacharias, acetic alcohol, 45; acetic carmine, 152; iron-carmine, 152.
Zacharias, E., chemistry of the cell, 359.
Zellner, natural injections, 311.
Zenker, fixing liquid, 42.
Zenthoefer, elastic tissue, 451.
Zernecke, preparation of *Ligula*, 476.
Ziegler, white cement, 290.
Ziehen, gold and mercury impregnation method, 434.
Ziehl's carbolic fuchsin, 196.
Zimmermann, microtomes, 73.
Zimmermann, sieve-dishes, 4; bone, 453.
Zimmermann, A., chemistry of the cell, 359.
Zinc, chloride, for hardening brain, 396, 397; impregnation method, 434.
Zinc-white cement, 290.
Zoantharia, 489.
Zoja, methylen blue, 204; bioblasts of Altmann, 368; *Hydra*, 490; Protozoa, 499.
Zoobothrium, 468.
Zoological methods, 466 *et seq.*
Zschokke, deltapurpurin, benzopurpurin, 225; cartilage, 456.
zur Strassen, *Bradynema*, 480.
Zwaardemaker, safranin stain, 190.

ERRATA.

Page 7, line 20, for "side" read "slide."
Page 68, line 3, for "cannel" read "cinnamon."
Page 459, line 2 from bottom, for Löwitz" read "Löwit's."

PRINTED BY ADLARD AND SON,
BARTHOLOMEW CLOSE, E.C., AND 20, HANOVER SQUARE, W.

UNIVERSITY OF CALIFORNIA LIBRARY,
BERKELEY

THIS BOOK IS DUE ON THE LAST DATE
STAMPED BELOW

Books not returned on time are subject to a fine of 50c per volume after the third day overdue, increasing to $1.00 per volume after the sixth day. Books not in demand may be renewed if application is made before expiration of loan period.

JUL 13 1927

AUG 10 1927

NOV 29 1927

MAR 24 1928

APR 7 1928

JUN 10 1930

50m-8,'26

QH231
L4
1896

71213

UNIVERSITY OF CALIFORNIA LIBRARY